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THE ANALYTICAL AND PHARMACOLOGICAL CHARACTERIZATION OF α-BENZYL-N-METHYLPHENETHYLAMINE, AN IMPURITY IN ILLICIT METHAMPHETAMINE SYNTHESIS

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at The Medical College of Virginia, Virginia Commonwealth University.

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However, I would mostly like to thank all of the people from the Departments of Pathology and Pharmacology that allowed me to invade their laboratories and time for no other reason than that they were good friends and colleagues: Dr. John Woodward, his graduate student and my good friend and classmate, Tooraj, and his great technologist, Tana Blevins for help with all of the NMDA and dopamine work; Dr. Dave Compton and his technologist, Kevin Jordan, Drs. Aron Lichtman and Eddie Ishac for their invaluable help with experimental design and statistical analysis of the *in vivo* pharmacology studies; most importantly, my fellow classmates John Shacka, Marissa Bernstein, and Ray Archer for the moral and party support that makes all the rest of it tolerable. And, of course, I would like to thank the United States Air Force for picking up the tab.

This work is dedicated to the good Lord above, that, for reasons only He knows, continues to give me the strength and ability to explore new adventures.. It's been said life is like a game that we must play with no knowledge of the object, the rules, or what constitutes "winning" or "losing". We must trust that God has some reason for the hand He deals us. In the academic sense, we are like students studying for a final exam, finding that as time winds down, we're not sure just what's important and what's not. In the words of Robert Frost: "I am no longer concerned with good and evil. What concerns me is whether my offering will be acceptable." In His honor, I will play these cards to the best of my ability and hope that this offering is acceptable.

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LIST OF ABBREVIATIONS

α_1AR	α_1 adrenergic receptor
AMPH	Amphetamine
B_{max}	Maximum amount of drug bound
BNMPA	α-Benzyl-N-Methylphenethylamine
Ci	Curie
CD_{50}	Convulsive dose, 50%
CFT; "WIN-35428"	2-β-carbomethoxy-3-β-(4-fluorophenyl)tropane-1,5-napthalene disulfonate
CNS	Central nervous system
d	Dextrorotatory; (+)-isomer
DEA	Drug Enforcement Administration
DP2P	Diphenyl-2-propanone
DP2P-OH	Diphenyl-2-propanol
DA	Dopamine
GABA	Gamma-amino-butyric acid
GC/MS	Gas chromatography/mass spectrometry
GEPR	Genetically epilepsy-prone rats
^3H	Tritium label
HFBA	Heptafluorobutyric anhydride
5-HT	5-hydroxytryptamine; serotonin
HI	Hydriodic acid

HPLC	High performance liquid chromatography
IC_{50}	Inhibitory concentration, 50%
IRS	Infrared spectroscopy
K_i	Inhibitory affinity constant
l	Levorotatory; (-)-isomer
LD_{50}	Drug dose required to produce lethality in 50% of the animals
4-MAX	4-methylaminorex
MDA	3,4-Methylenedioxyamphetamine
MDMA	3,4-methylenedioxymethamphetamine
METH	Methamphetamine
mM	Millimolar
mV	Millivolts
µA	Microamps
µM	Micromolar
nA	Nanoamps
nM	Nanomolar
NA	Norepinephrine
N-demethyl-BNMPA	N-demethyl-α-benzyl-N-methylphenethylamine
NMDA	N-methyl-D-aspartate
NMR	Nuclear magnetic resonance
pM	Picomolar
p-OH-BNMPA	para-hydroxy-α-benzyl-N-methylphenethylamine
p-OH-N-demethyl-BNMPA	para-hydroxy-N-demethyl-α-benzyl-N-methylphenethylamine

P2P	Phenyl-2-propanone
PPA	Phenylpropanolamine
PRZ	Prazosin
PXT	Paroxetine
SD	Standard deviation
SEM	Standard error of the mean
TFMPP	1-[3-(trifluoromethyl)phenyl] piperazine
TPH	Tryptophan hydroxylase
V_d	Apparent volume of distribution

Abstract

THE ANALYTICAL AND PHARMACOLOGICAL CHARACTERIZATION OF
α-BENZYL-N-METHYLPHENETHYLAMINE,
AN IMPURITY IN ILLICIT METHAMPHETAMINE SYNTHESIS

By Karla Adeline Moore, Ph.D.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at The Medical College of Virginia, Virginia Commonwealth University.

Virginia Commonwealth University, 1995

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Methamphetamine (METH) is a popular drug of abuse, readily synthesized in clandestine laboratories. Illicit synthesis results in various contaminants, one of which is α-benzyl-N-methylphenethylamine (BNMPA). This dissertation investigates the hypotheses that contaminants like BNMPA may contribute to the toxicity of METH and that detection of BNMPA/metabolites in biological fluids may be utilized as markers of illicit METH consumption.

Based on metabolic studies of benzphetamine (a structurally similar compound), we predicted the four major metabolites of BNMPA to be N-demethyl-BNMPA , diphenyl-2-propanone , para-hydroxy-N-demethyl-BNMPA , para-hydroxy-BNMPA , and diphenyl-2-propanol. We synthesized these compounds and developed a gas chromatography/mass spectrometry detection

method. We confirmed these as true metabolites in humans following ingestion. para-Hydroxy-BNMPA and para-hydroxy-N-demethyl-BNMPA (as conjugates) were the major metabolites detected .

The utility of these compounds as markers of illicit methamphetamine consumption was confirmed when BNMPA metabolites were found in two of eighty METH-(+) urine samples. Additonally, a trace amount of para-hydroxy-BNMPA was detected in the urine from a patient who died following METH consumption.

BNMPA's contribution to METH toxicity was investigated in mice where it produced convulsions without affecting spontaneous locomotor activity or altering METH-induced increases in spontaneous activity or METH-induced convulsions.

The *in vitro* effects of BNMPA on a variety of neuronal receptor types was determined. BNMPA and N-demethyl-BNMPA displaced a dopamine transporter selective ligand (^{3}H-CFT) from rat striatal membranes. BNMPA ($\geq$ 100 μM) enhanced basal efflux of tritiated dopamine from striatal slices yet had no effect on tritiated norepinephrine efflux from hippocampal slices. BNMPA also fully inhibited the binding of the serotonin transporter selective ligand ^{3}H-paroxetine to cortical membranes. BNMPA fully displaced the α_1-receptor selective ligand ^{3}H-prazosin from whole brain membranes. BNMPA significantly inhibited NMDA-activated currents in oocytes expressing the NR1/2A or NR1/2C receptors but did not alter GABA induced currents in cultured cortical neurons. The BNMPA-induced blockade of NMDA currents was voltage-dependent with increased blockade at more negative potentials. These results suggest that BNMPA may have multiple sites of action in the CNS that could be important in modulating the behavioral effects of methamphetamine contaminated with this byproduct.

Chapter 1

Review of Literature and Rationale for Study

1.1 General introduction

"Medicine sometimes cures, it often relieves, it always consoles" (Anonymous). The use of drugs and therapeutics to "cure", "relieve" and "console" is as ancient as the art and science of medicine itself. It is often the ability of a chemical to "console" rather than to "cure" which has led to its ultimate abuse. Human societies, in general, have historically had a strong and pervasive commitment to the use of drugs (Cooper, 1979). This seems to be especially true of those drugs which exert potent effects upon the central nervous system (CNS) .

Archaeological studies at sites several thousand years old have uncovered the use of psychoactive substances by several methods: poppy-seed caches in Turkey, statues demonstrating ritual drug enemas in South America, and incised poppy capsules on the headdresses in ancient Crete (Westermeyer, 1988). As history has dawned in various regions, a wide variety of psychoactive substances have come into use. Beer and wines were obtained from various agricultural products, including mammalian milk, in East Asia, Europe and Africa. Cactus wines and beers were prepared by Central American and southwestern North American tribes. Various uses of opium have been described from ancient

Egypt to China. The recreational use of stimulants to relieve the boredom of day-to-day agricultural work has been reported from India to Southeast Asia to Malaysia and the rest of Oceania (Ahluwalia, 1968). The historical masters of developing new substances seem to be the aboriginal inhabitants of the New World who developed over 200 psychoactive substance (including such favorites as coca leaf, tobacco, and peyote) from leaves, roots, barks cacti and vines (DuToit, 1977). Because crop stores were too limited to permit excessive use, chronic substance abuse was probably rare or non-existent until the agricultural revolution in the centuries leading up to the 1500's.

During the 1600 and 1700's, gardening and farming evolved to larger and more complex operations and it became possible to manufacture alcohol in quantities sufficient for abuse (Westermeyer, 1988). "Cash cropping" in psychoactive substances appeared during this era. Not coincidentally, this is also when classical substance abuse disorders such as delirium tremens as a complication to alcohol dependence and opiate withdrawal were first described. Legal restrictions against excessive use of drugs also appeared about this time.

When long-distance, relatively efficient sea commerce developed in the 1700 and 1800's, societies were exposed to new substances and to new methods of drug administration. Additionally, technological advances such as distillation further modified and enhanced the impact of psychoactive substances. The first epidemics of psychoactive substance abuse, most notably the Gin Epidemic in England, occurred during this era. Fetal alcohol syndrome was first described during this time. Opium smoking appeared and increased exponentially from the seventeenth to the nineteenth centuries. In Japan and China, these national epidemics have required centuries to subside and, in fact,

continue today in Burma, Laos and Thailand (Lau, et al.,1967; Singer, 1974; Westermeyer, 1976).

The Industrial Revolution of the 1800's stimulated an explosion of organized production of psychoactive substances such as opium, tea, coffee, betel-areca, and grains and grapes for alcohol products. Scientific and technological advances of the twentieth century have fostered the ability to delineate the exact nature and structure of the active component of psychoactive compounds leading to the ability to synthesize them completely in the laboratory without requiring a plant-based compound at all. Among the first of these were the barbiturates and amphetamines, followed later by synthetic opioids, hallucinogens and "sedatives" such as the benzodiazepines (Westermeyer, 1988).

Amphetamine was first synthesized by Edelano in 1877 (Lee, et al., 1992). During the 1930's, Prinzmetal, Bloomberg, and others first used amphetamines clinically as a central nervous system stimulant for the treatment of narcolepsy and depression where its abuse potential first became evident (Prinzmetal and Bloomberg, 1935). Since that time, the ability of amphetamines to alleviate fatigue, improve performance of simple mental and physical tasks, elevate mood, increase confidence and produce euphoria has led to their misuse and abuse.

Amphetamine use (here to include amphetamine, methamphetamine, phenmetrazine, methylphenidate, diethylproprion and propylhexedrine) reached epidemic proportions during the late 1940's and early 1950's when these compounds were used by soldiers, factory workers and prisoners of war in Japan during World War II. After World War II, a surplus on the Japanese market permitted sales without a prescription, with peak use occurring about 1954 (Brill

and Hirose, 1969). In the 1960's, methamphetamine abuse became a social problem in the United States. By 1970, 50% of legally manufactured amphetamine and related compounds were being sold illegally on the black market.

Increasing abuse of these drugs led to their classification as a Schedule II controlled substance under the Controlled Substances Act (Public Law 91-513) of 1970, limiting methamphetamine's acquisition through legitimate channels. This Act stringently regulated the manufacture of these stimulants and forced manufacturers to decrease sales to retail pharmacies (Morgan and Kagan, 1978). Because methamphetamine is easily synthesized even in crude laboratories, it quickly became the "stimulant of choice", and a dramatic increase has occurred in the illicit production and use of methamphetamine hydrochloride over the past several years. Endemic areas for this increase include the Pacific coast states, Hawaii and other Pacific rim countries such as Japan and Korea (Heischober and Derlet, 1989).

During the decades in which amphetamines were used primarily for medical purposes and were available through legitimate manufacturing channels, these drugs were considered remarkably safe and only rarely responsible for death (Kalant and Kalant, 1975). Prior to instituting the Controlled Substances Act in 1970, the world medical literature contained only 43 reports of deaths associated with amphetamines in a 35-year period. As with any legally manufactured drug, these compounds are subject to strict manufacturing and purification requirements and would not be expected to contain a significant amount of impurities. However, since 1980, as clandestinely manufactured compounds have become the primary source of

amphetamine/methamphetamine, these compounds have consistently ranked among the twenty most frequently mentioned drugs in emergency room patients as well as medical examiner cases (DAWN, 1993). It is well known that side reactions and incomplete conversions ("impurities of manufacture") can easily occur in most of the illicit synthetic methods and "street chemists" rarely, if ever, take the time or expense to purify their product. In fact, the addition of even more diluents and adulterants is the norm (Morgan and Kagan, 1978). Impurities of manufacture are numerous and are characteristic of a particular synthetic method. These contaminants have been extensively reviewed elsewhere (van der Ark, et al., 1978; Sinnema and Verweij, 1981; Verweij, 1989; Soine, 1989). This thesis discusses the analytical and pharmacological characterization of one of these contaminants, α-benzyl-N-methylphenethylamine (BNMPA).

Knowledge of impurities is important for several reasons: 1) impurities could have additional harmful effects on the methamphetamine user; 2) knowledge of impurities can provide useful intelligence to law enforcement officials since some impurities are a result of only one particular synthetic method; 3) information on new synthetic methods, including necessary chemicals and equipment, may be obtained; 4) with knowledge of materials being used, law enforcement officials can monitor production and sale of commercially available precursors of methamphetamine synthesis; 5) the presence or absence of specific impurities can aid in identifying samples which are of common origin and in distinguishing between samples of legitimate and illicit manufacture;
6) awareness of impurities is important to the forensic chemist performing sample analysis because of possible interference with the analytical technique (Barron,

et al., 1974); and 7) identifying an impurity or its metabolites in biological fluids may serve as a marker of illicit methamphetamine consumption.

Little work has been done to characterize these compounds *in vivo*. Chemically, the compounds have been well described. Bailey, et al. (1974), Sinnema, et al. (1981), Huizer, et al. (1985), Noggle, et al. (1985), and Soine (1986) have described various methods of synthesis of the phenalkylamines and their related impurities of manufacture. Thin-layer chromatographic methods for the detection of impurities have been described by Barron, et al. (1974), van der Ark, et al. (1978), and Sinnema, et al. (1981). High performance liquid chromatography (HPLC) methods have been described by and Noggle, et al. (1985) and Lambrechts, et al. (1986) . Nuclear magnetic resonance (NMR) data has been published by Barron, et al. (1974), Bailey, et al. (1974), Kram, et al. (1977), van der Ark, et al. (1978), Sinnema, et al. (1981), and Huizer, et al. (1985). Infrared spectroscopy (IRS) studies have been carried out by Bailey, et al. (1974), Barron, et al. (1974), Huizer, et al. (1985) and Noggle, et al. (1985). Capillary gas chromatography/nitrogen phosphorus detection has been described by Tanaka, et al. (1994) while gas chromatography/mass spectrometry (GC/MS) characterization has been done by Bailey, et al. (1974), Barron, et al. (1974), Lomonte, et al. (1976), Kram, et al. (1977), Sinnema, et al. (1981), Huizer, et al. (1985) and Noggle, et al. (1985).

Depending on the synthetic route used and the skill of the chemist, impurities of manufacture may range from 3 to 30 % of the sample (Soine, 1986). Long-time abusers in whom tolerance to the drug has developed may use as much as 5,000 to 15,000 mg of methamphetamine per day (Derlet and Heischober, 1990) and consequently may be consuming these impurities in

relatively high quantities. When one considers this in conjunction with the apparent increase in emergency room visits and medical examiner cases since illicit manufacture became the primary source of methamphetamine, it is surprising that the information concerning the pharmacology of the impurities of manufacture is very limited. The work that has been done is summarized in Table 1. There is no literature describing the activity of the remaining impurities of manufacture. However, even these limited studies indicate that the α-benzyl compounds with convulsive doses, 50%(CD_{50}) much less than their lethall doses, 50% (LD_{50}) cause greater CNS stimulation at the brainstem and cord levels than that seen with amphetamine/methamphetamine. The potential danger of street drugs containing substantial amounts of these impurities is evident and further enforces our rationale for the biological characterization of BNMPA.

1.2 Synthesis and detection of α-benzyl-N-methylphenethylamine

Laboratories producing methamphetamine comprised more than 50% of all laboratories seized by the Drug Enforcement Administration (DEA) during a 45-month period ending in September, 1981 (Frank, 1983). The most popular method of synthesis at that time (over 50% of labs seized), probably because of its ease and economy, was reductive amination using phenyl-2-propanone (P2P), methylamine, aluminum foil, mercuric chloride catalyst, and alcohol. In the next most popular method (less than 10%), the product of an acetaldehyde/methylamine reaction was refluxed with benzylmagnesium chloride. The third most popular method (less than 10%) used the Leukart

TABLE 1. PHARMACOLOGY / TOXICOLOGY OF IMPURITIES FOUND IN ILLICITLY SYNTHESIZED METHAMPHETAMINE

Compound	Species	End Point	Dose (mg/kg)	Route of Administration	Reference
2-(phenylmethyl)phenethylamine	Rabbits	Lethality (LD_{50}) (Hypotension)	198.5	Continuous i.v. infusion	Moisset de Espanes, et al. (1953)
" "	"	"	31.7	Single i.v. dose	" "
" "	"	"	160	Subcutaneous	Hano and Wojewodzki(1961)
" "	Man	Death	Unknown	Unknown	Anonymous (1981)
Phenyl-2-propanone	Mice	Loss of righting ability (LRA_{50})	215	Intraperitoneal	Barfknecht et al. (1971)
"	"	LD_{50}	520	"	" "
Phenyl-2-propanol	Mice	LRA_{50}	330	"	" "
"	"	LD_{50}	540	"	" "
BNMPA	Mice	Convulsions (CD_{50})	54.09	Intraperitoneal	Noggle et al. (1985)
"	"	LD_{50}	78.2	"	" "
α-benzylphenethylamine	Mice	CD_{50}	45.49	"	" "
"	"	LD_{50}	63.25	"	" "
d,l-Amphetamine sulfate	Mice	LD_{50}	7	Subcutaneous	Nielsen et al. (1967)
"	"	CD_{50}	90	Intraperitoneal	Noggle et al. (1985)
"	"	LD_{50}	91.14	"	" "
d,l - Methamphetamine HCl	Mice	CD_{50}	56.96	Intraperitoneal	" "
"	"	LD_{50}	57.19	"	" "

reaction, refluxing P2P with either methylamine and formic acid or N-methylformamide with hydrochloric acid.

An important precursor in these syntheses is P2P which, until 1981, was available commercially. Because of its importance in illicit synthetic methods,it is now listed as a Schedule-II controlled substance with some clandestine laboratories now synthesizing just P2P. The most common synthetic by-product present in P2P prepared from phenylacetic acid is diphenyl-2-propanone (DP2P) (Soine, 1986). When P2P containing this contaminant is used to synthesize methamphetamine via reductive amination routes, BNMPA is a major contaminant (Figure 1) (Barron, et al., 1974).

During the late 1970's and early 1980's, the conversion of ephedrine to methamphetamine by reductive cleavage of the hydroxyl group either using thionyl chloride or hydriodic acid (HI) and red phosphorous was relatively uncommon (10 laboratories) (Soine, 1986). In fact, through the late 1980's, DEA reports contained significantly more mentions of laboratories which were synthesizing methamphetamine using one of the reductive amination methods or synthesizing P2P. However, with the growing difficulty in obtaining precursors for the more popular reductive amination routes, and the increasing availability of (-)-ephedrine and (+)-pseudephedrine both in this country and the Far East, methamphetamine synthesized from ephedrine has "flooded" the market (*Microgram*, 1994).

In response to this change in synthetic methods, the DEA has sought to increase regulations governing transactions in bulk ephedrine (Federal Register, 1994). Lack of availability and price increases in precursors such as HI has made violators seek alternative sources of HI and/or different processes of

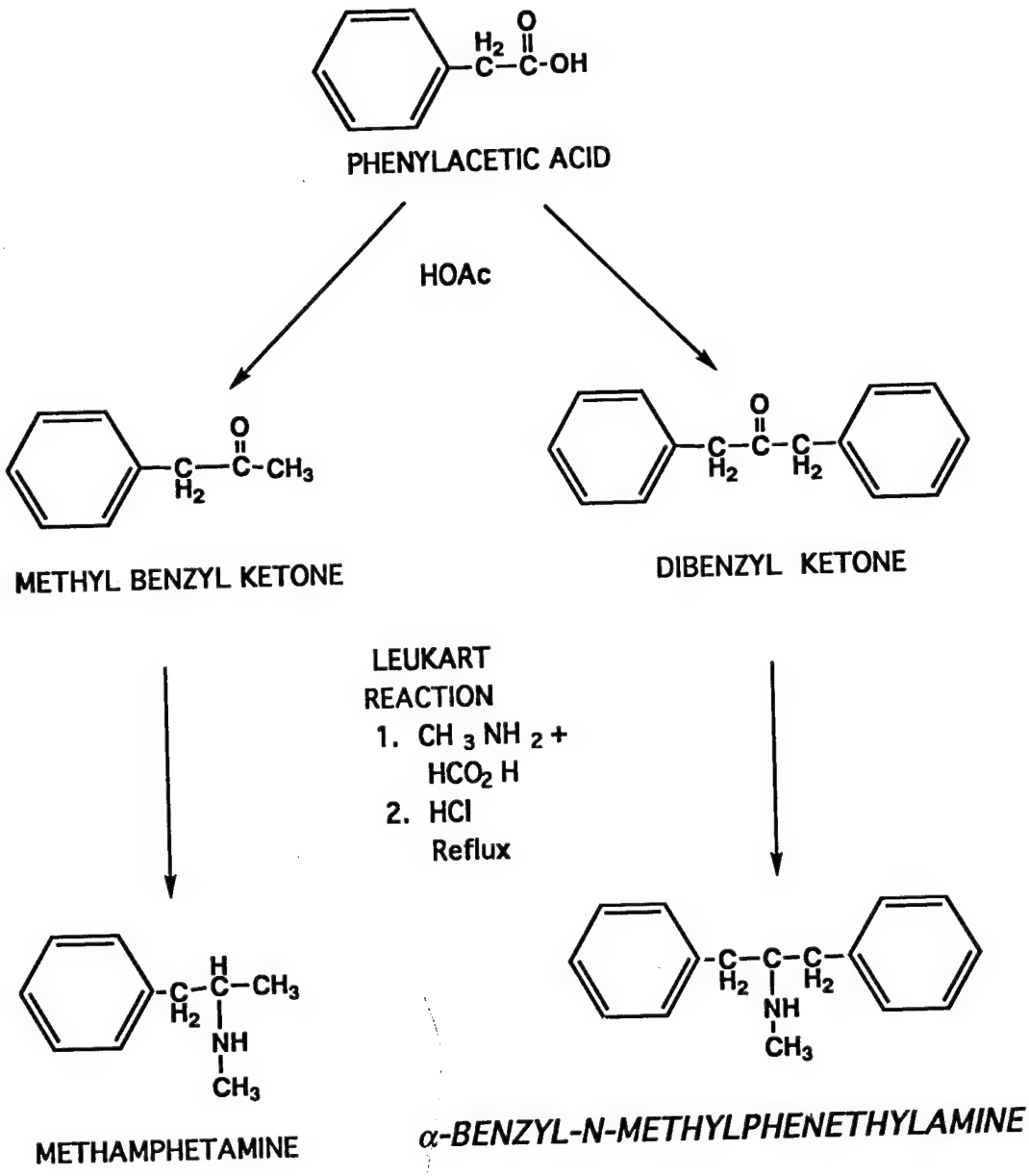


FIGURE 1. ORIGIN OF α-BENZYL-N-METHYLPHENETHYLAMINE FROM PHENYLACETIC ACID AND THE LEUKART REACTION
-Barron, et al. (1974). J. Assoc. Off. Anal. Chem. 57:5

clandestinely manufacturing methamphetamine (*Microgram,* 1993). It is very likely that there will be a resurgence in the use of the reductive amination methods for the production of methamphetamine and an increased visibility of impurities such as BNMPA over the next decade.

Two reductive amination methods will be used to synthesize BNMPA: one will use aluminum foil as the reducing agent in the identical procedure used by street chemists to synthesize methamphetamine. Instead of P2P, DP2P will be used. If the recovery of pure product is insufficient for further studies using this method, this will at least establish that this impurity can arise via this synthetic method. The second procedure will use a modification of the method developed by Borch (1971) using sodium cyanoborohydride as the reducing agent.

1.3 Metabolism of α-benzyl-N-methylphenethylamine

Hepatic microsomal enzymes are responsible for the metabolism of most drugs. The plasma, kidney, lung and gastrointestinal tract can also contribute to the biotransformation process. Drug metabolism is classified into "Phase-I" and "Phase-II' reactions. In general, phase-I reactions convert the compound to more polar metabolites by oxidation, reduction or hydrolysis while Phase-II reactions involve coupling of either the parent compound or the Phase-I metabolites with an endogenous compound such as glucuronides or various acids (sulfuric, acetic or amino) (Benet and Schneider, 1985).

Glucuronides constitute the majority of Phase-II metabolites of phenols, alcohols, and carboxylic acids (Benet and Schneider, 1985). Glucuronide formation is catalyzed by various liver microsomal glucuronyltransferases. Some Phase-I metabolism and conjugations other than glucuronide formation are

catalyzed by non-microsomal enzymes. Nonmicrosomal biotransformation also occurs primarily in the liver. Inactivation of aromatic primary amines and hydrazines involves conjugation with acetic acid. Aromatic carboxylic acids are often inactivated by conjugation with glycine. Conjugation with glutathione is not a quantitatively important route of elimination but it does contribute to the inactivation of several highly toxic epoxide intermediates produced by hydroxylation. Other nonmicrosomal conjugates include sulfate conjugation of phenols and O-, S-, and N- methylation of amines and phenols.

Based on this information and studies in humans of benzphetamine metabolism (Figure 2) (Inoue and Suzuki, 1986), the four major metabolites of BNMPA were predicted to be the N-demethyl compound, p-OH-BNMPA, p-OH-N-demethyl BNMPA and diphenyl-2-propanone (DP2P) (Figure 3). Additionally, it was expected that these metabolites would be excreted primarily as glucuronide or sulfate conjugates and a hydrolysis step was included in the detection method.

1.4 Acute toxicity and pharmacology

The definition of a drug is necessarily very broad, and can refer to any chemical substance that affects living processes. "Drugs", therefore, include synthetic chemicals, natural products and foodstuffs (Cooper, 1992). Here, BNMPA is included in the definition. A drug interaction (between two or more drugs) can be defined as the modification of the effect of one drug by another, whether that modification be enhancement or antagonism (Cooper, 1992). In the case of drugs of abuse, and specifically CNS stimulants such as METH, the primary concern is the enhancement/antagonism of toxic effects. The

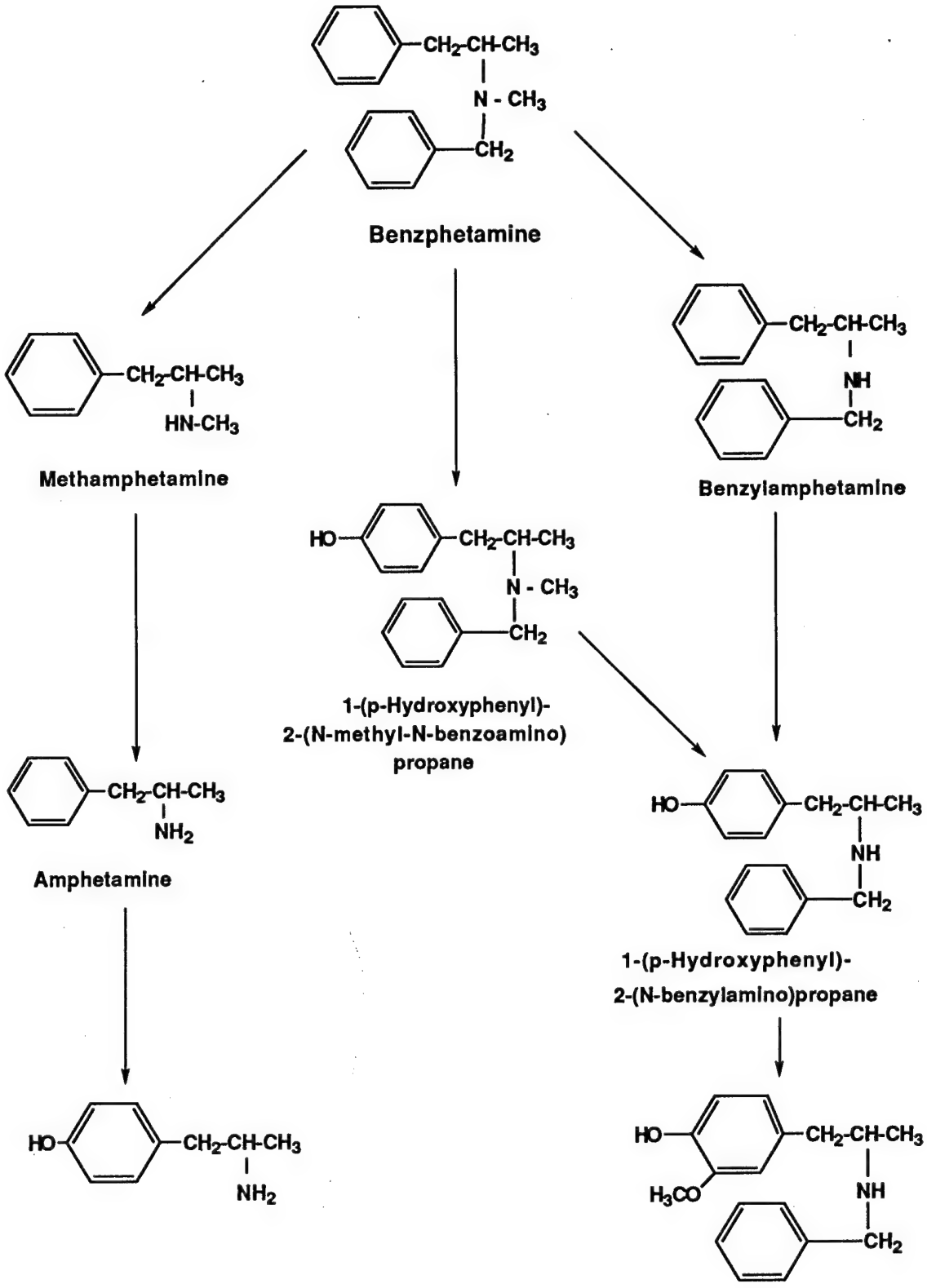


FIGURE 2. METABOLISM OF BENZPHETAMINE

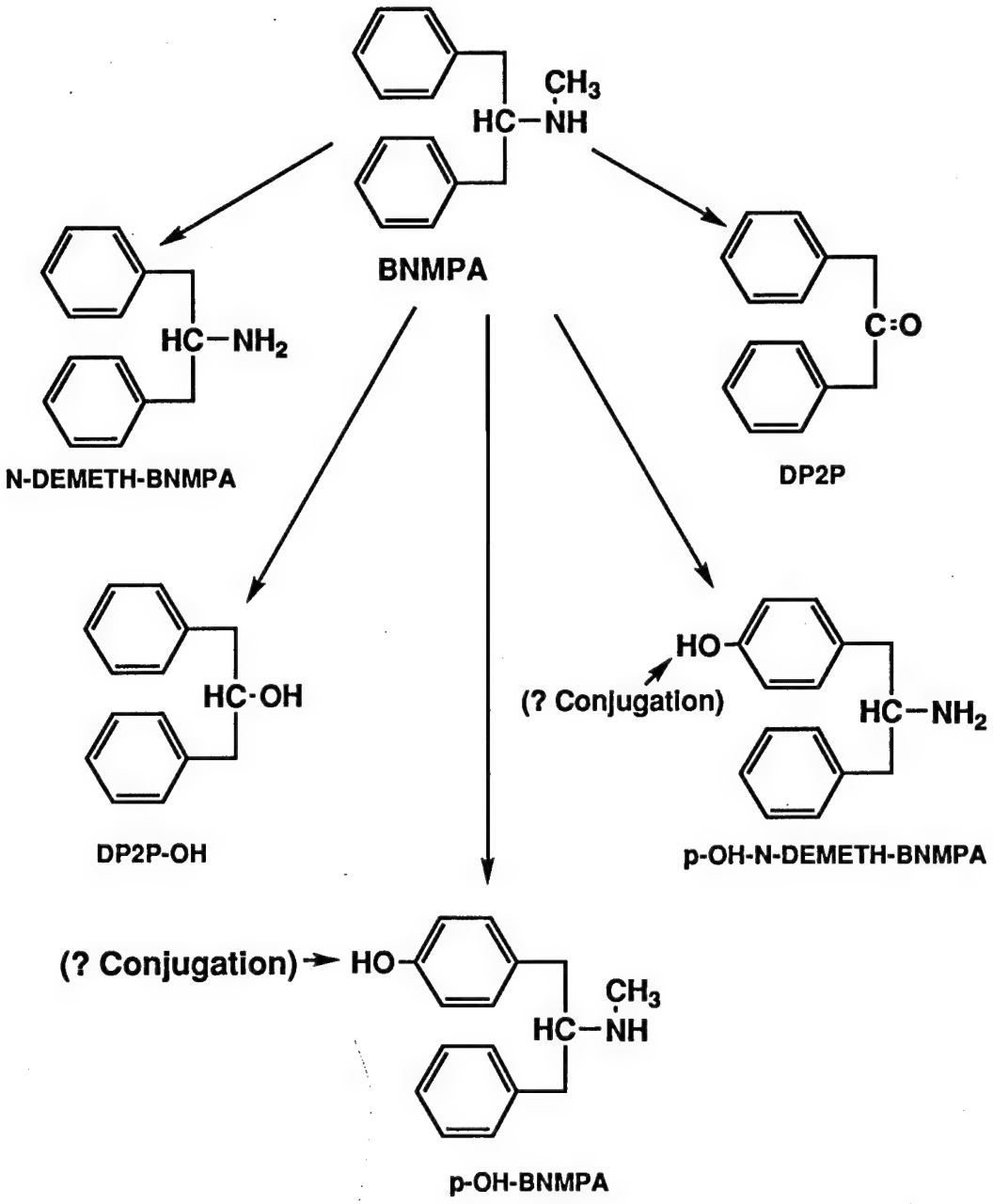


FIGURE 3. PREDICTED METABOLITES OF
α-BENZYL-N-METHYLPHENETHYLAMINE (BNMPA)

combinations of effects of the drugs being taken may complicate the signs and symptoms of intoxication, making it more difficult to identify the drugs being used and to design a plan of care.

One of the defining behavioral characteristics of psychomotor stimulants (METH, AMPH, cocaine, etc.) is their ability to elicit increases in motor activity. At low doses, these drugs produce an alerting response characterized by increases in exploration, locomotion, grooming, and rearing. As the dose is increased, locomotor activity decreases and behavior patterns become stereotyped, i.e. a continuous repetition of one or several types of behavior. Although the specific behaviors differ across species, these behaviors are components of the species natural repertoire, but are performed in an abnormal repetitive pattern. In their review of the behavioral effects of psychostimulant drugs, Robbins and Sahakian, (1983) stated that speculation in the early 1900's was that the locomotor response was primarily a noradrenergic (NE) response However, Creese and Iverson (1975) found little effect of specific NE depletion on either locomotion or stereotopy. The locomotor response was later found to depend on DA release in the nucleus accumbens (mesolimbic DA system). The majority of evidence now indicates that the neurochemical effects of these stimulants underlying their ability to increase motor activity involve dopaminergic systems (Johanson and Fischman, 1989). By a variety of means, including enhancement of neurotransmitter release and blockade of reuptake, these drugs facilitate catecholaminergic neurotransmission. The "rewarding effects" believed responsible for psychomotor stimulant abuse are also thought to result from DA release in the nucleus accumbens (Robbins and Sahakian, 1983).

However, the production of stimulant stereotopies, while still a dopaminergic response, is believed to be due to alterations in the nigro-striatal pathways. The role of striatal dopamine in the production of stimulant stereotopies is permissive rather than causal and reflects a qualitative change in the manner in which nigro-striatal neuronal function influences the interaction of dopamine with its postsynaptic receptor. At low doses of drug, when nigro-striatal activity is still maintained, the release of DA into the cleft and the level of interaction between released transmitter and postsynaptic receptor is still regulated by the presynaptic neuron and any input the dopaminergic neuron receives from other systems. Thus, low doses of stimulants augment impulse-mediated DA release in the striatum or other dopaminergic areas and enhance behavioral output. As the dose of drug is increased, postsynaptic DA receptor activity becomes totally dissociated from the regulatory input of presynaptic firing and the postsynaptic cell receives random DA input that can no longer be processed. The constant bombardment of the DA receptor may also lead to a form of "tachyphylaxis" to the dopaminergic regulatory input. In either case, information transfer from the dopaminergic neuron to the postsynaptic cell is eliminated, and output from the striatum undergoes a qualitative change, no longer dependent on progressively increasing quantitative changes in DA hyperactivity. The appearance of focused stereotopy must therefore occur at the expense of enhanced locomotor activity (Kuczenski, 1983).

Seizures, another "toxic" event often associated with stimulant abuse, tend to occur only at very high doses (Weiner, 1985; Cameron, et al., 1992; Ritz and George, 1993). Two of the mechanisms drugs can use to increase excitability in the CNS (be "seizurgenic") are blocking inhibition or enhancing

excitation (Franz, 1985). The primary inhibitory neurotransmitter in the CNS is gamma-aminobutyrate (GABA). Many convulsants, whose actions remained unexplained for many years (e.g., pentylenetetrazol and picrotoxin), have been found to be selective antagonists of GABA (Bloom, 1985). Glutamate and aspartate are found in very high concentrations in the brain and both are very powerful excitatory neurotransmitters. Glutamate activation of the N-methyl-D-aspartate (NMDA) receptors leads to a rapid and significant increase in intracellular calcium. This elevation in intracellular calcium is believed to be the primary event in neuronal death observed in cell culture models of glutamate-mediated excitotoxicity (Choi, 1992).

Additionally, in studies with cocaine, Ritz and George (1993) demonstrated that distinct neuronal binding sites appear to be associated with the initiation of cocaine-induced seizures and lethality. They suggested that the potencies of cocaine and related drugs in producing seizures appear to be highly associated with drug affinity at serotonin transporters, whereas the potencies of these drugs in producing lethality appear to be most strongly associated with their affinities at dopamine transporters. This study also demonstrated that the affinity of cocaine and related compounds at sigma receptors or muscarinic cholinergic receptors appear to be related to an attenuation of both seizures and lethality. Their findings predicted that cocaine related drugs with relatively high affinity at the serotonin transporter and relatively low affinities at sigma and muscarinic receptors appear most likely to exhibit potent seizurgenic effects.

The mechanisms of seizure induction appear to be as varied as the drugs themselves, however, serotonin depletion has been implicated in several models of convulsions, neurotoxicity and clinical syndromes such as depression, multiple

sclerosis, schizophrenia and "stimulant psychosis" (Rudnick and Clark, 1993). Depletion of the amine in brain increases, whereas agents that increase 5-HT concentration decrease susceptibility to seizures (Przegalinski, 1994).

Depletion of 5-HT from all regions of the rat CNS by p-chlorophenylalanine or intracerebroventricular injections of 5,7-dihydroxytryptamine (5,7-DHT) has been shown to enhance the intensity and the incidence of tonic seizures by maximal electroshock (Browning, et al., 1978) and by sound (Jobe, 1981). Agents that suppress serotonergic transmission have been shown to increase both lidocaine and pentylenetetrazole-induced convulsions, whereas drugs that facilitate 5-HT function decreased the incidence of these convulsions (Endo, et al., 1993). Genetically epilepsy-prone rats (GEPR) are known to have deficiencies in both brain norepinephrine and 5-HT. When these rats are treated with carbamazepine and antiepilepserine (i.p.), significant increases in 5-HT are seen at the approximate time to peak anticonvulsant effect. Pretreatment of these same rats with p-chlorophenylalanine depleted brain 5-HT and greatly diminished the anticonvulsant effectiveness of both drugs (Yan, et al., 1992). Fluoxetine, an antidepressant and inhibitor of serotonin reuptake, has also been shown to be an effective anticonvulsant in GEPRs and a strong relationship has been shown between fluoxetine's effects on the serotonergic system and its anticonvulsant effect (Dailey, et al., 1992). Decreases in 5-HT concentrations in various regions of the brain have also been correlated to increased aggressive behavior caused by fenitrothion (Anand, et al., 1989) as well as seizures induced by lindane (Sunol, et al., 1988). Prostaglandins of the E series are known to exert anticonvulsant actions in experimental animals. PGE1 has been shown to inhibit PTZ-induced convulsions in rats through a serotonin-mediated mechanism.

PGD2 has also been shown to produce a dose-related inhibition of PTZ-induced clonic convulsions. This anticonvulsant action of PGD2 was significantly attenuated following pretreatment with pharmacological agents known to reduce central serotonergic activity, including 5,6-dihydroxytryptamine (a selective serotonergic neurotoxin), p-chlorophenylalanine (a specific inhibitor of serotonin biosynthesis), metergoline (a serotonin postsynaptic receptor antagonist), and quizapine, which is known to inhibit neuronal release of serotonin (Bhattacharya and Parmar, 1987).

This research pursues the pharmacology and toxicology of BNMPA alone and with METH, in particular the two major toxic events associated with stimulants described above, i.e. increases in sponatneous activity and proconvulsant capabilities. It is important to consider the interaction of BNMPA with METH since, as an impurity of METH manufacture, BNMPA is ingested with METH and would rarely, if ever, be consumed by itself.

1.5 Interaction of α-benzyl-N-methylphenethylamine at central nervous system receptors

Because BNMPA has been shown to be seizurgenic at doses much lower than lethality (Noggle et al., 1985), it was predicted that it would display antagonist-like activity at the GABA receptor, agonist-like activity in the NMDA pathway, some type of interference in serotonin pathways or a combination of all of these effects. Because of its similarity to benzphetamine, BNMPA's structure should predispose it to also have a low affinity for the dopaminergic receptors responsible for locomotor activity and a greater affinity for serotonergic or other "seizurgenic" receptors. Like its structurally analogous anorexiants, BNMPA may

inhibit TPH activity or be a 5-HT_{1C} or 5-HT_2 receptor antagonist. Additionally, BNMPA may show significant, specific affinity for the α_1-receptor. If it is acting as an antagonist at this site, this activity would contribute to its convulsant nature.

1.6 Rationale and objectives of study

Despite the declining availability of pharmaceutically synthesized amphetamines, a dramatic increase has occurred in the use of METH over the past several years. In one county in California, METH intoxication was cited in 40% of all drug-related homicides (a 52% increase over the previous year) (Bailey and Shaw, 1987). In an inpatient adolescent drug treatment unit in California, METH was listed as the drug of choice by about 80% of patients due to METH's wide availability, low cost, and longer duration of action when compared to cocaine (Heischober and Derlet, 1989). Because of this increased demand, illicit production of METH has also increased. Illicitly synthesized METH may be contaminated by any number of compounds ranging from lead (Allcott, et al., 1987) to other stimulants/adulterants used as diluents to impurities of manufacture such as BNMPA. It should not be surprising, then, that the number of coroners cases involving METH use has risen to twice that of cocaine (Puder, et al., 1989) despite METH and AMPH's historically high therapeutic index (Kalant and Kalant, 1975). What is surprising is the relatively small amount of work that has been done on the effects of these impurities of manufacture in biological systems.

The goal of this research project is to characterize the pharmacology and toxicology of α-benzyl-N-methylphenethylamine (BNMPA), one of the impurities of illicit methamphetamine manufacture. BNMPA is often seen from manufacture

utilizing the Leukart reaction or simple reductive amination using aluminum foil and mercuric chloride.

Characterization will include synthesis of the "pure" impurity as well as the predicted metabolites. Identification of these compounds will be accomplished with GC/MS and nuclear magnetic resonance (NMR). Perfection of these techniques for identifying this substance will be necessary for identification and "tracking" of this substance through *in vivo* systems.

Following confirmation that the "pure" product has been synthesized, studies will begin on the pharmacological properties of this compound. Predicted metabolites will be confirmed in humans following ingestion of BNMPA as well as screening of METH/AMPH positive human forensic urine samples.

Since BNMPA is only ingested in conjunction with illicit methamphetamines, such a unique marker would be of great benefit to the forensic community in identifying abusers as well as helping to pinpoint sources of illegal methamphetamine manufacture. By elucidating the structure or identifying the presence of contaminants, forensic chemists are able to determine the method of synthesis. This information may be used to limit the availability of precursors, to determine the substance being synthesized (even if synthesis is incomplete), and to group seizures to common sources of production.

Acute toxicity and other possible stimulant-like activity will be characterized in mice. These observations will be confirmed at the molecular level using *in vitro* assay systems for various CNS receptors/transporters.

Chapter 2

General Methodology

2.1 Chemicals and reagents

DP2P was purchased from Eastman Chemical Company (Rochester, New York). All other chemicals were purchased from Aldrich Chemical Company. Solvents were HPLC grade, purchased from Fischer Scientific Products/Fischer Scientific (Fair Lawn, New Jersey). Heptafluorobutyric anhydride (HFBA) was purchased from Regis Chemical Company (Morton Grove, Illinois). d-Amphetamine-d_3 sulfate and β-glucuronidase (Type H-2, from *Helix pomatia*) were purchased from Sigma Chemical Company (St. Louis, Missouri). d-Amphetamine-d_3 sulfate was used as the internal standard for all GC/MS procedures.

BNMPA, N-demeth-BNMPA and DP2P-OH were synthesized as described in Chapter 3. p-OH-BNMPA was a gift of Richard Glennon, Ph.D. and Abd Ismaiel, Ph.D., Department of Medicinal Chemistry, Medical College of Virginia. The structure, as the oxalate salt, (m.p.=180-182 °C.) was confirmed in their laboratory with proton nuclear magnetic resonance (NMR). We further confirmed the structure of the underivatized and HFBA-derivatized compound in our laboratory with GC/MS.

2.2 Extraction, derivatization and hydrolysis

2.2.1 Non-hydrolyzed samples

Two ml of urine containing 500 ng/ml d-amphetamine-d3-sulfate (3.6 µM) as internal standard and any other analyte of interest was alkalinized with concentrated ammonium hydroxide. The analytes were extracted with 4 ml of n-butyl chloride. n-Butyl chloride was chosen as the extraction solvent because of its tendency for "clean extractions" and infrequency of emulsion formation. It was also hoped that the search for these impurities could be combined with routine amph/meth confirmation procedures and n-butyl chloride is the extraction solvent of choice in many liquid/liquid extraction procedures. Following mixing and centrifugation, 3 ml of the n-butyl chloride layer was transferred to a 12 x 75 borosilicate test tube and reduced in volume to 2 ml. under a gentle stream of nitrogen at room temperature. One hundred microliters (100 µl) of HFBA were added and heated at 70^{o} C for 20 minutes. The n-butylchloride was then evaporated under nitrogen and the residue resuspended in 50 µl ethyl acetate. Two microlitres were injected into the GC/MS (Yamamoto, et al., 1989). The derivatized compounds were confirmed by NMR and GC/MS. In the SIM mode, mass ions194, 193 and 91 were monitored for DP2P-OH; 119, 91 and 210 for DP2P; ions 194, 316 and 103 for the N-demethyl compound; 330, 194 and 133 for BNMPA; 406, 316 and 303 for p-OH-N-demethyl BNMPA; and ions 330, 406 and 542 for p-OH-BNMPA (Fig.4-9). Retention times were 6.8, 6.9, 7.7, 8.1, 8.8 and 9.0 minutes, respectively (Fig. 10). For quantitation, the ratios of the194 (DP2P-OH), 119 (DP2P), 194 (N-demeth), 330 (BNMPA), 406 (p-OH-N-demeth) and 330 (p-OH BNMPA) ions to the deuterated amphetamine ion 243 were monitored.

2.2.2. β-glucuronidase hydrolysis

Two and one-half ml acetate buffer (pH 5) and 400 µl β-glucuronidase were added to 4 ml of sample (patient, control or calibrator) containing 250 ng/ ml (1.8 µM) amphetamine-d3 in a disposable, screw cap test tube. These samples were capped, vortexed and allowed to incubate at 55° C for 6 hours. Samples were cooled and 5 ml of 50% potassium phosphate dibasic buffer (pH 11) were added. The analytes were extracted with 4 ml. of n-butyl chloride and derivatized as described above.

2.2.3 Acid hydrolysis:

One ml of 37% (concentrated) HCl was added to 4 ml of sample (patient, control or calibrator) in a disposable, screw cap test tube containing 250 ng/ml amphetamine-d3. Samples were capped, vortexed and hydrolyzed for 20 minutes in a steam autoclave at 125° C. Samples were then cooled to room temperature. One and one-half ml concentrated NH_4OH were added to each to achieve a final pH =10. Extraction and derivatization were carried out as before.

2.3 Instrumentation

GC/MS analysis was performed on a Hewlett-Packard 5890 GC equipped with a 12 m. x 0.2 mm(id) x 0.33 µm HP-1 capillary column connected to a Hewlett-Packard 5971-A mass selective detector. Data processing was performed with a HP Chemstation (Version 3.2 software). The GC/MS was operated in the splitless mode with a helium carrier gas linear velocity of 20 ml/min. Initial oven temperature was 100 °C with an injection port temperature of 250 °C. The temperature program was ramped from an initial temperature of 100 °C (held for 2 minutes) to 280 °C at 20 °C/minute.

Nuclear magnetic resonance (NMR) studies were performed in $CDCl_3$ (Aldrich Chemical Company) on a "GE-NMR QE-300". Melting points were determined on a Thomas-Hoover Capillary Melting Point Apparatus-"Uni-Melt" (Arthur H. Thomas Co., Philadelphia, PA).

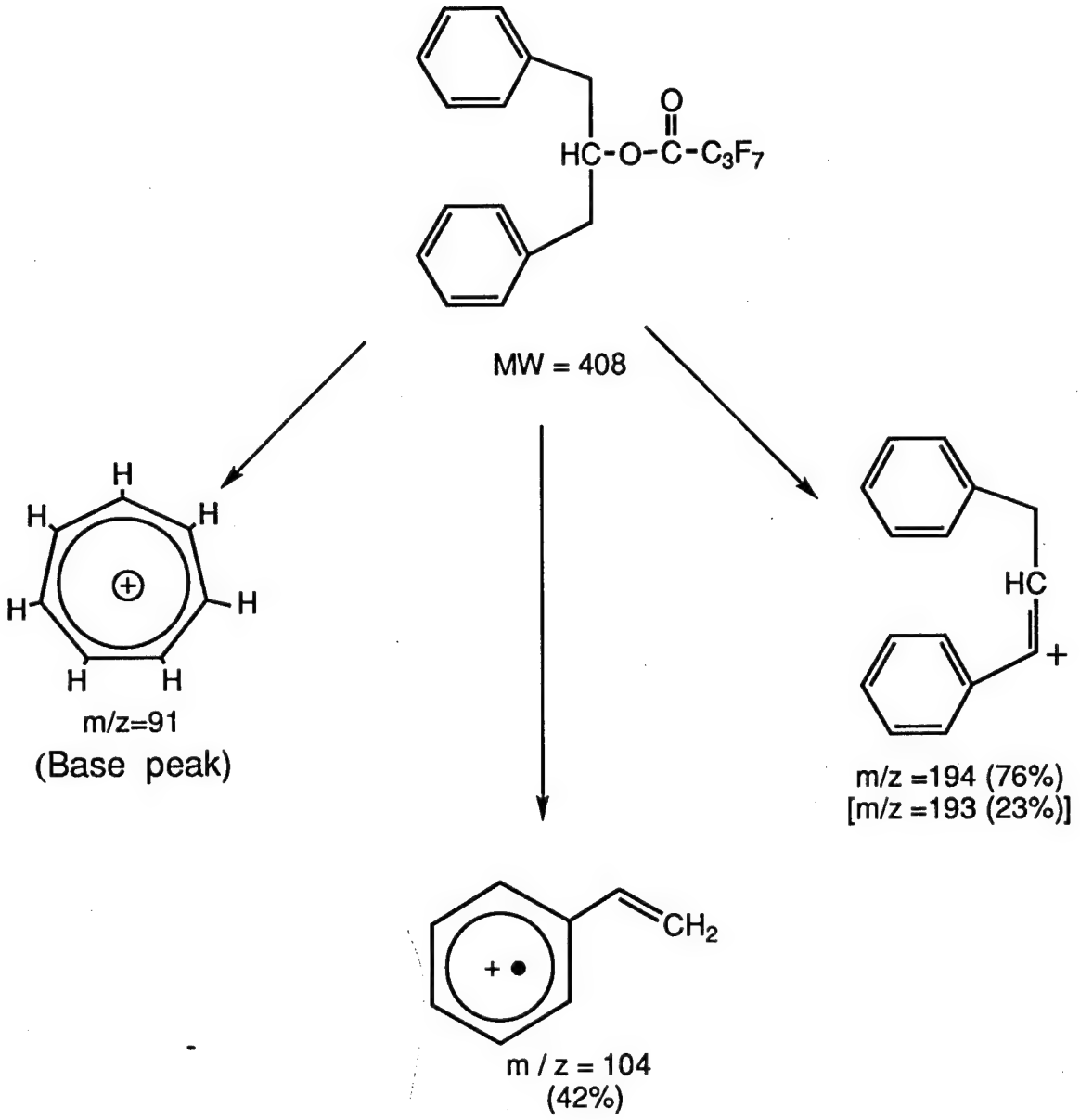


FIGURE 4. DIPHENYLPROPANOL (DP2P-OH) : HFBA
ELECTRON IMPACT FRAGMENTATION

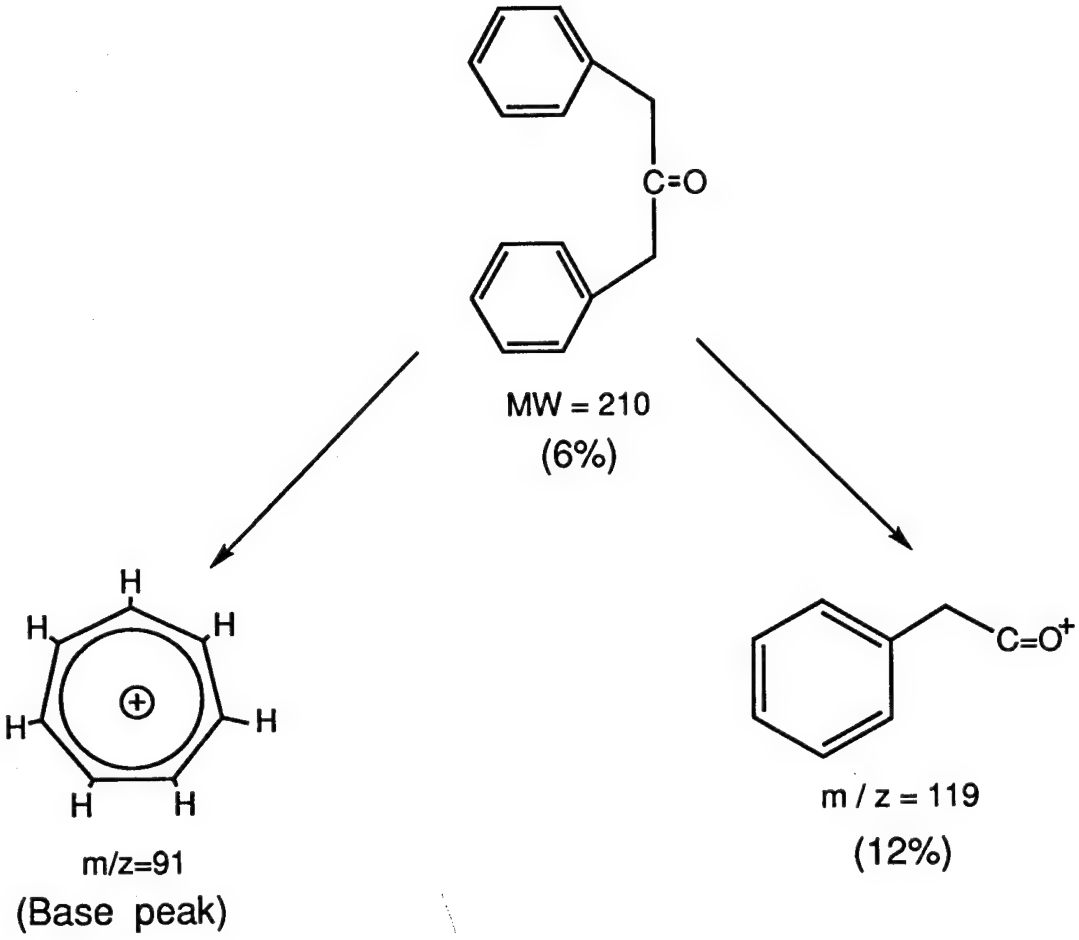


FIGURE 5. DIPHENYLPROPANONE (DP2P)
ELECTRON IMPACT FRAGMENTATION

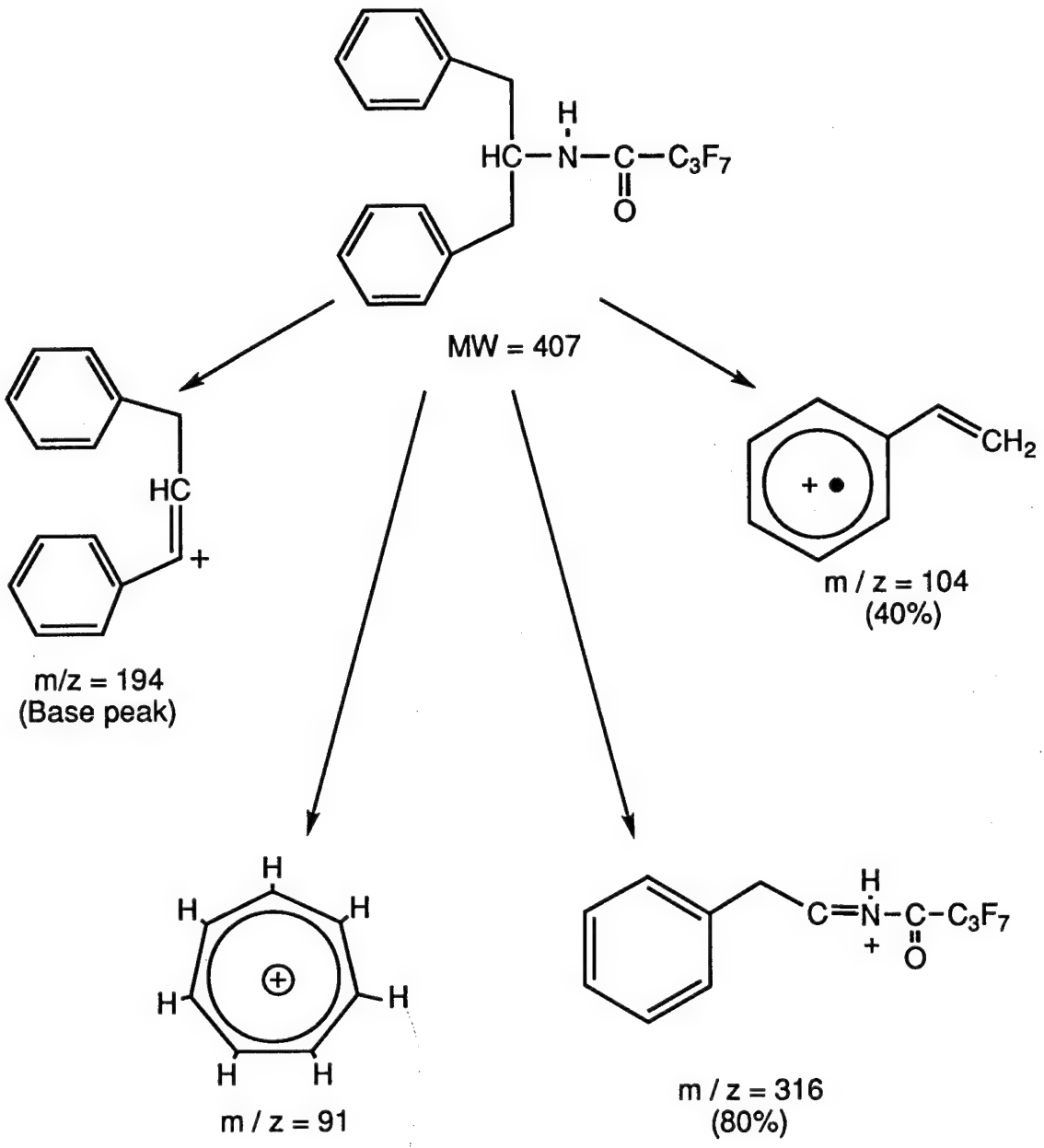


FIGURE 6. N-DEMETHYL-α-BENZYL-N-METHYLPHENETHYLAMINE : HFBA ELECTRON IMPACT FRAGMENTATION

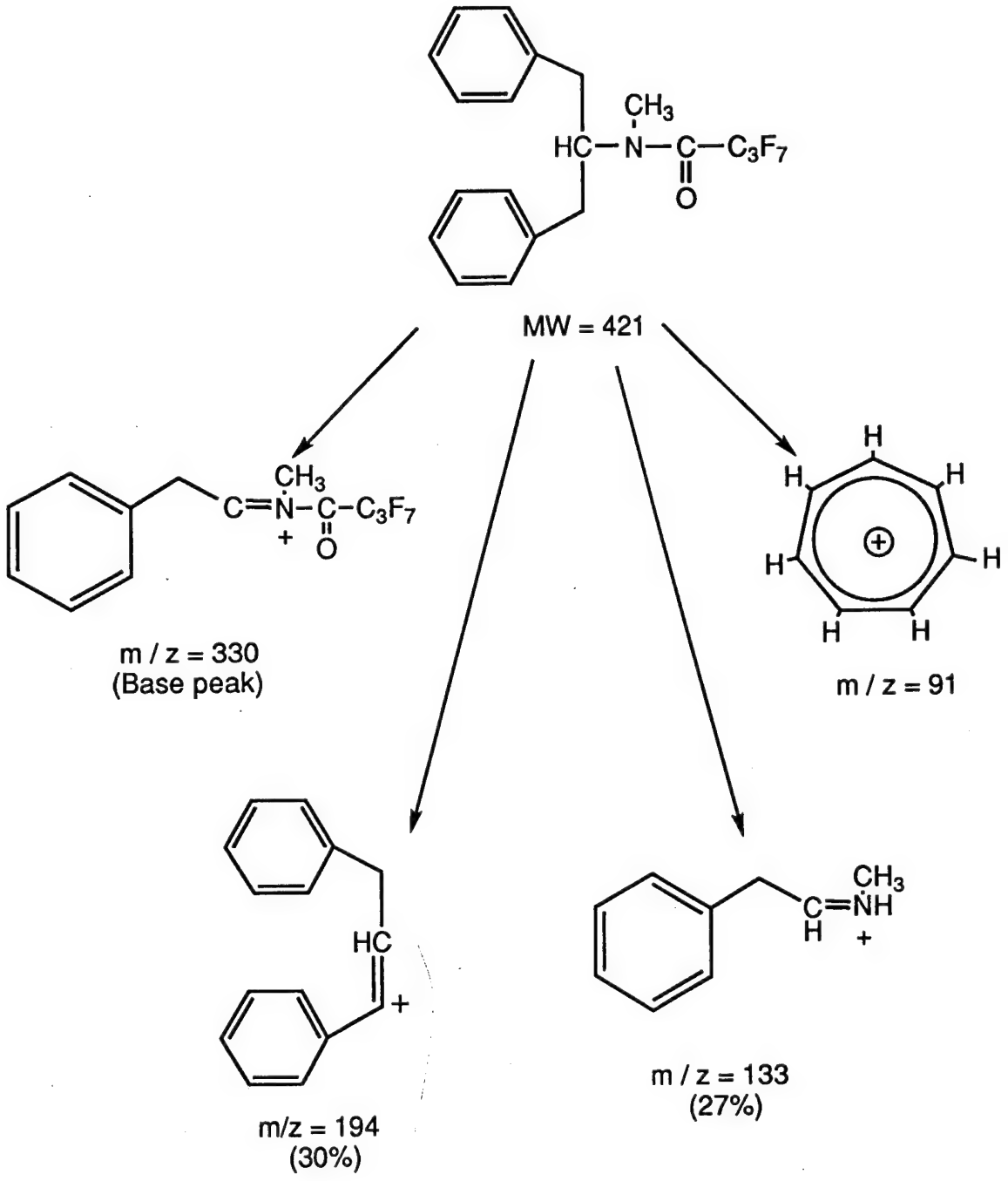


FIGURE 7. α-BENZYL-N-METHYLPHENETHYLAMINE (BNMPA) : HFBA ELECTRON IMPACT FRAGMENTATION

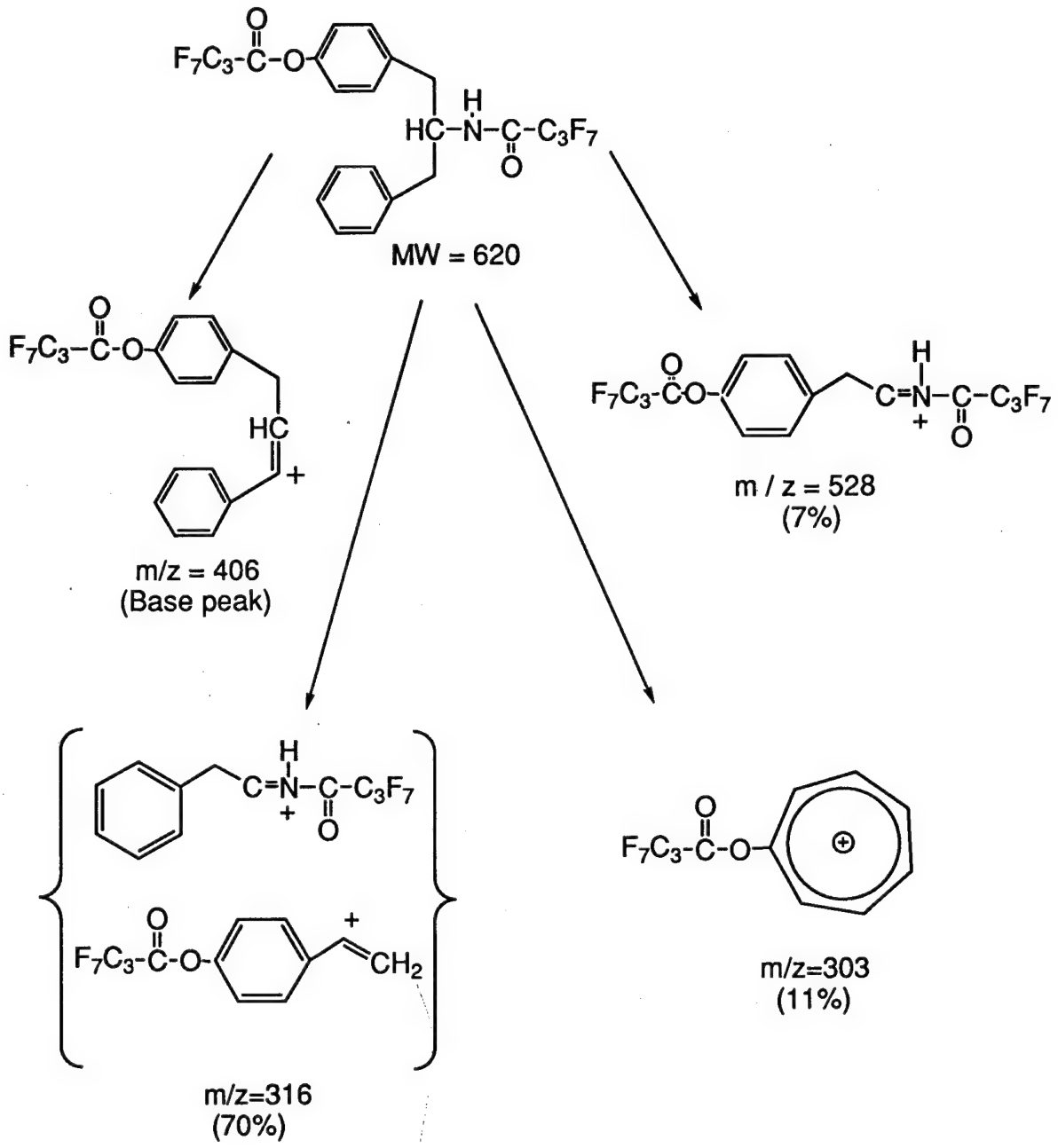


FIGURE 8. p-OH-N-DEMETHYL-α-BENZYL-N-METHYLPHENETHYLAMINE : HFBA ELECTRON IMPACT FRAGMENTATION

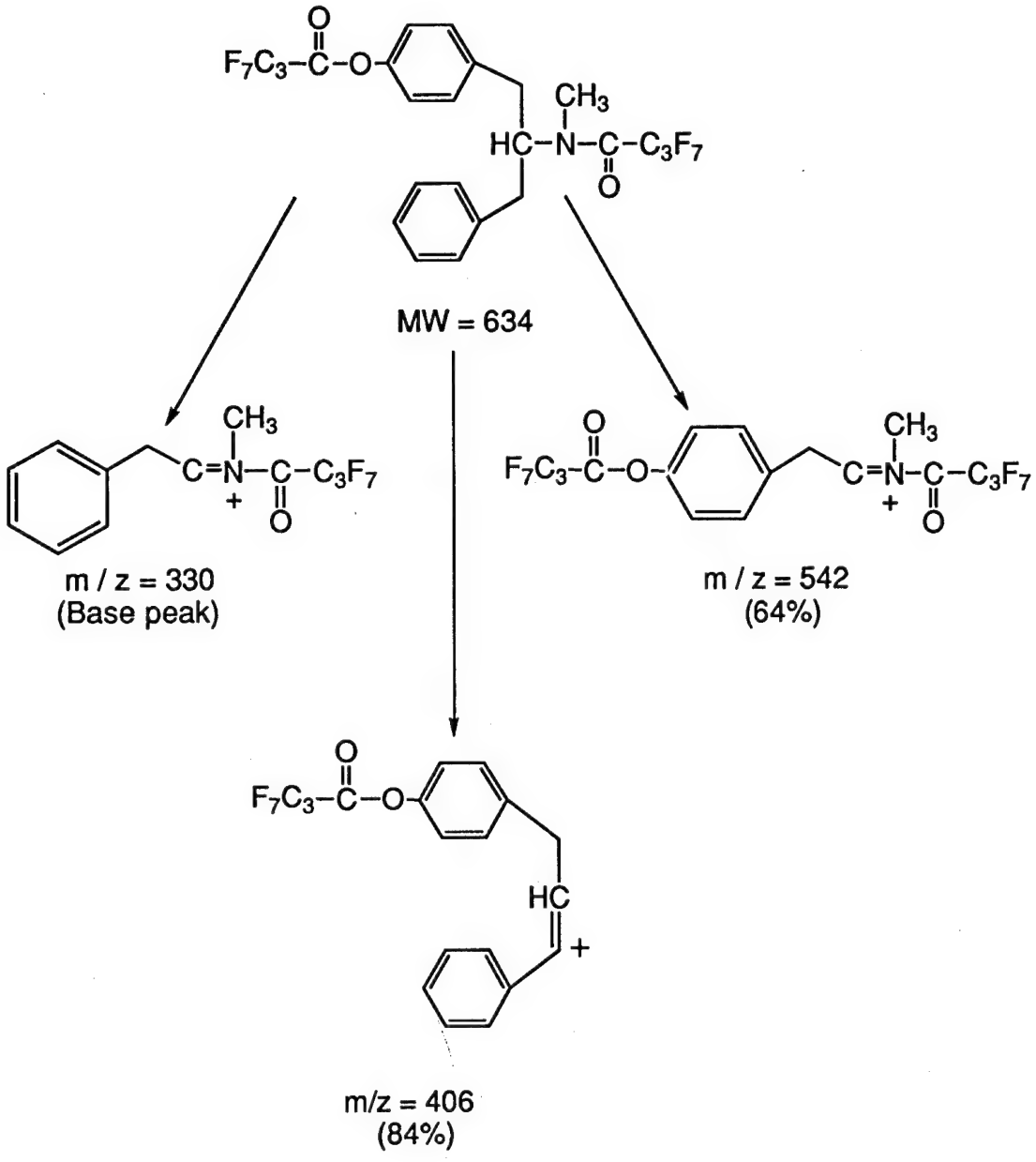


FIGURE 9. p-OH-α-BENZYL-N-METHYLPHENETHYLAMINE : HFBA
ELECTRON IMPACT FRAGMENTATION

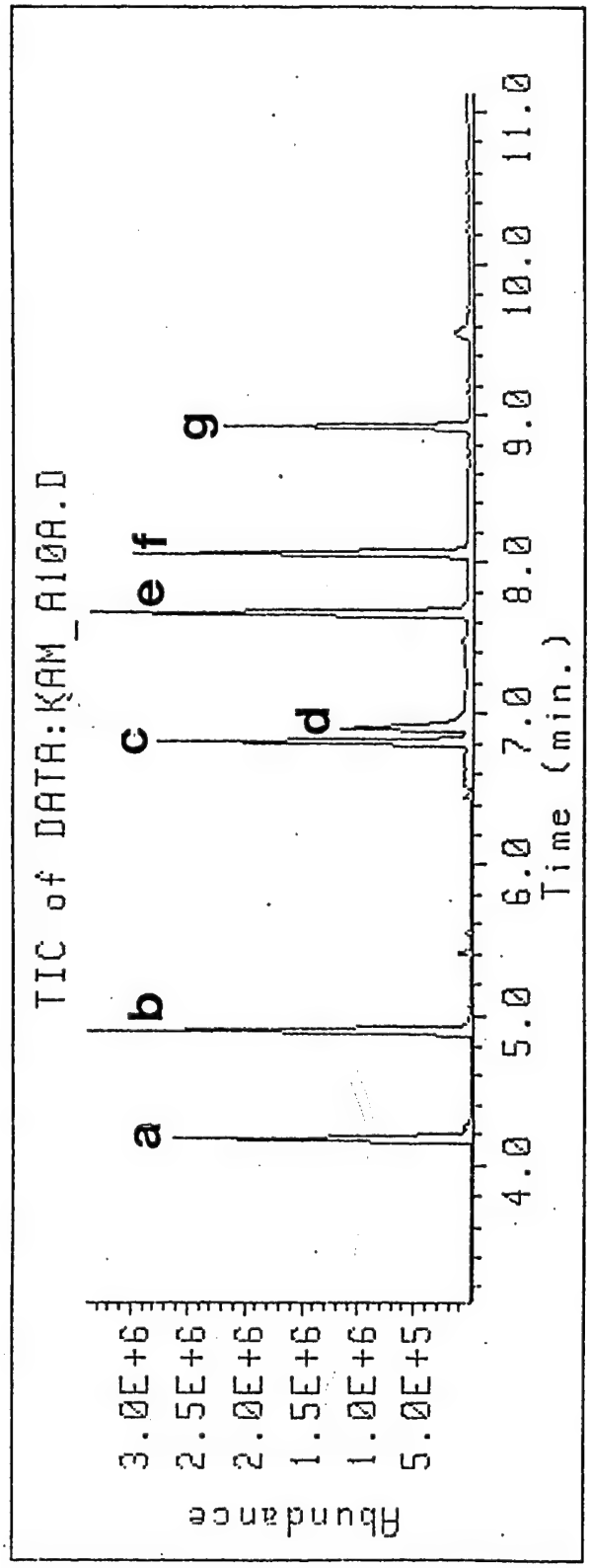


FIGURE 10. TOTAL ION CHROMATOGRAM OF BNMPA AND METABOLITES
a = AMPHETAMINE - d3; b = METHAMPHETAMINE -d5; c = DP2P - OH;
d = DP2P; e = N-DEMETHYL-BNMPA; f = BNMPA; g = p-OH-BNMPA

Chapter 3

Synthesis and GC/MS Analysis of α-Benzyl-N-methylphenethylamine (BNMPA) and Anticipated Metabolites in Human Urine

3.1 Abstract

α-Benzyl-N-methylphenethylamine (BNMPA) is an impurity of illicit methamphetamine synthesis. We synthesized BNMPA and three of its anticipated metabolites: N-demethyl-α-benzylphenethylamine, diphenyl-2-propanone and diphenyl-2-propanol. The purity and structure of these compounds and their heptafluorobutyric anhydride (HFBA) derivatives were confirmed by melting point, GC/MS and nuclear magnetic resonance (NMR). A GC/MS method to detect these compounds in urine, using liquid/liquid extraction and derivatization with HFBA was developed. Interference studies showed BNMPA and its proposed metabolites to be well-resolved from other common phenethylamine drugs and HHS-FUDT required analytes. The limit of detection for BNMPA and metabolites was 2.5 ng/ml (13 nM); the limit of quantitation for the four compounds was 25 ng/ml (130 nM). The calibration curves were generally linear from 25 to 500 ng/ml (130 nM - 2.6 μM) . Typical within run CV's (at the LOQ) ranged from 13% to 20% (n=8). Between run CV's over one month at 25 ng/ml were 9 to 28% and at 500 ng/ml were 2.6 to 3.9%. The detection of BNMPA or its metabolites in urine samples may provide a marker of use of illicitly synthesized methamphetamine.

3.2 Introduction

Impurities that arise from the illicit synthesis of drugs of abuse are generally not removed by the "street chemist" from the final product which is ultimately distributed to and ingested by the abuser. Since these impurities may have additional harmful effects on the user, we were interested in characterizing the pharmacology/toxicology of α-benzyl-N-methylphenethylamine (BNMPA), an impurity of illicit methamphetamine synthesis. Additionally, since some impurities are a result of only one particular synthetic method, we were interested in the possibility of using the presence of an impurity or its metabolites in biological fluids as a marker of consumption of clandestinely synthesized methamphetamine.

Because little work has been done with these compounds *in vivo*, it was first necessary to predict the metabolites of this compound, synthesize these metabolites and develop a reliable method for their detection in biological fluids.

Deamination, N-demethylation, oxidative deamination, hydroxylation and conjugation are common metabolic pathways for most licit and illicit drugs (Baselt and Cravey, 1989). BNMPA and its N-demethyl "metabolite" were synthesized using Noggle's method (1985). Synthesis of the alcohol, 1,3-diphenyl-2-propanol, was a simple ketone reduction using sodium borohydride (Mancera, et al., 1953). The final anticipated metabolite, 1,3-diphenyl-2-propanone, was purchased. We predicted other metabolites could include p-hydroxylated BNMPA and p-OH-N-demethyl BNMPA but these compounds were not synthesized for this method development. The p-OH metabolites were, however, considered in screening of patient urine samples.

A detection method for these compounds in urine using gas chromatography/mass spectrometry (GC/MS) with heptafluorobutyric anhydride (HFBA) derivatization is also described. Method development included determination of sensitivity, selectivity, lower limit of detection (LOD) and lower limit of quantitation (LOQ). Interference studies with other phenethylamines and Health and Human Services (HHS) required analytes (Federal Register, 1994) were accomplished.

3.3 Methods

3.3.1 BNMPA synthesis-Aluminum as reducing agent

This method was a modification of the method published by Wassink et al. (1974) and is a common method used in the synthesis of illicit methamphetamine (Glennon, 1989). We used DP2P as the starting material instead of phenylacetone and methylamine·HCl instead of ammonia. Methylene chloride was used as the extraction solvent.

3.3.2 BNMPA Synthesis - Sodium borohydride as reducing agent

BNMPA and its N-demethyl derivative (α-benzylphenethylamine) were synthesized using a slight modification of the method of Noggle (1985). NMR spectra and melting points were consistent with Noggle's data.

3.3.3 Synthesis of 1,3-diphenyl-2-propanol

Two grams (10 mmole) DP2P were combined with 0.3 g sodium borohydride in 250 ml methanol. This mixture was stirred at room temperature for one hour. The excess sodium borohydride was destroyed with 1-2 drops of glacial acetic acid. The methanol was evaporated in vacuo. The residue was suspended in 25 ml diethyl ether and washed with 3 x 25 ml deionized water. The

ether extract was dried over magnesium sulfate and filtered. The ether was evaporated in vacuo leaving a pale yellow oily residue. This was confirmed to be diphenyl propanol (DP2P-OH) by NMR and GC/MS.

3.3.4 Calibrators

Calibrators of 6.25, 12.5, 25, 50, 100, 200 and 500 ng/ml were prepared from a 1 mg/ml (in methanol) stock standard of each compound. The 500 and 200 ng/ml calibrators were prepared by diluting 50 and 20 µl, respectively, of each stock standard to 1 ml methanol, making 50 and 20 µg/ml working standards, respectively. Twenty µl of each of these solutions were added to 2 ml of urine, resulting in the final calibrator concentration of 500 and 200 ng/ml urine. The remaining working standards of 10, 5, 2.5, 1.25 and 0.62 µg/ml were prepared as serial dilutions of the 20 µg/ml working standard. Twenty µl of each of the resulting dilutions were added to 2 ml. of urine to make the 100, 50, 25, 12.5 and 6.25 ng/ml calibrators.

3.3.5 LOD/LOQ determination

Lower limits of detection (LOD) and lower limits of quantitation (LOQ) were determined for BNMPA and its 3 proposed metabolites by both the statistical and empirical methods. The statistical method is well described by Anderson (1989) and consists of testing a series of blank samples and determining the mean "blank value" and standard deviation (SD). We tested 6 blanks and set the LOD at the mean plus 3 times the SD and the LOQ at the mean plus 10 times the SD.

The empirical (experimental) method consists of analyzing serial dilutions of the analytes. The LOD is the lowest concentration at which the results still satisfy some predetermined acceptance criteria (Armbruster, et al., 1994). Needleman and Romberg (1990) have used this method to determine the LOD's

for GC/MS assays for abused drugs. We used serial dilutions from 500 ng/ml to 2.5 ng/ml to determine LOD/LOQ by this method. LOD was defined as the concentration at which all routine GC/MS acceptance criteria (retention time within 2% of the calibrator and ion ratios within 20% of the calibrator) are met at least 90% of the time. LOQ was defined as the concentration at which all of the above criteria were met and the quantitative value was ±20% of the target concentration. Additionally, 3 runs per day on 4 separate days were made of this series of samples to determine the within run and between run coefficient of variance (CV). CV values were calculated from the cumulative mean and SD values [CV = (SD/mean) x 100].

3.3.6 Interference studies

Using the derivatization and extraction procedure described in Chapter 2, 500 ng/ml of BNMPA and each of its proposed metabolites were combined with 500 ng/ml of each of the following compounds: β-phenethylamine, d-amphetamine-d3, d-amphetamine, phentermine, phenylpropanolamine (PPA), d,l-methamphetamine-d5, d-methamphetamine, ephedrine, pseudephedrine, phenylephrine and benzphetamine.

Separation of BNMPA and its metabolites from cocaine (100 ng/ml), benzoylecgonine (1000 ng/ml), phencyclidine (50 ng/ml), morphine (250 ng/ml), codeine (250 ng/ml), tetrahydrocannabinol carboxylic acid (100 ng/ml), and 6-monoacetyl-morphine (200 ng/ml) was also studied.

3.4 Results

Synthesis of BNMPA using aluminum as the reducing agent produced 0.9 g (0.004 mol) BNMPA for an 11% yield. While this yield was much too low to

produce the amount of BNMPA we would need to complete future studies, it did confirm that this impurity could be produced from this common illicit synthetic method.

Using sodium borohydride as the reducing agent, the average production of BNMPA for 3 "runs" was 1.9 g (0.008 mol) , a 32% yield. Two "runs" of the procedure to synthesize the N-demethyl compound produced 4.0 grams (0.02 mol) for a 20% yield.

Using the statistical method of method analysis, the LODs for BNMPA, N-demethyl BNMPA and DP2Propanol were 3.4, 7.3 and 0.3 ng/ml, respectively. The LOQs were 4.1, 18.3, and 1.1 ng/ml, respectively. We felt these values were unrealistically low for a GC/MS procedure and re-established LODs and LOQs using the empirical method. Empiricallly, the LOD for BNMPA and metabolites was 2.5 ng/ml; the LOQ for the four compounds was 25 ng/ml. The calibration curves were generally linear from 25 to 500 ng/ml. Typical within run CV's (at the LOQ; n=8) for BNMPA, N-demethyl BNMPA, DP2P, and DP2Propanol and between run CV's over one month at 25 and 500 ng/ml are given in Table 2.

All of the phenethylamine (Figure 11; Table 3) and HHS-FUDT analytes (Table 4) were well resolved from BNMPA and its metabolites either by retention time, ions monitored or both.

3. 5 Conclusions

This method development lays the foundation for our *in vivo* studies, specifically the ability to identify either the impurity or a unique metabolite in the urine of suspected abusers. The presence of these compounds in biological fluids may provide confirmation that the methamphetamine administered by these

individuals was obtained illicitly. This information, in turn, may provide information to law enforcement officials on possible sources of the illicit compounds.

TABLE 2. WITHIN RUN AND BETWEEN RUN COEFFICIENTS OF VARIATION (CV) OF BNMPA AND METABOLITES

COMPOUND	WITHIN RUN (ng / ml)		BETWEN RUN (25 ng / ml)		BETWEEN RUN (500 ng / ml)	
	Mean ± SD	%CV	Mean ± SD	%CV	Mean ± SD	%CV
DP2PROPANOL	24.1 ± 5.8	14	27.8 ± 5.7	20	498 ± 17	3.5
DP2P	35.6 ± 4.4	12	28.9 ± 10.9	37	501 ± 20	3.9
N-DEMETH BNMPA	28.8 ± 6.0	21	25.0 ± 7.2	28	497 ± 18	3.7
BNMPA	25.2 ± 4.4	17	24.8 ± 2.3	9	492 ± 13	2.6

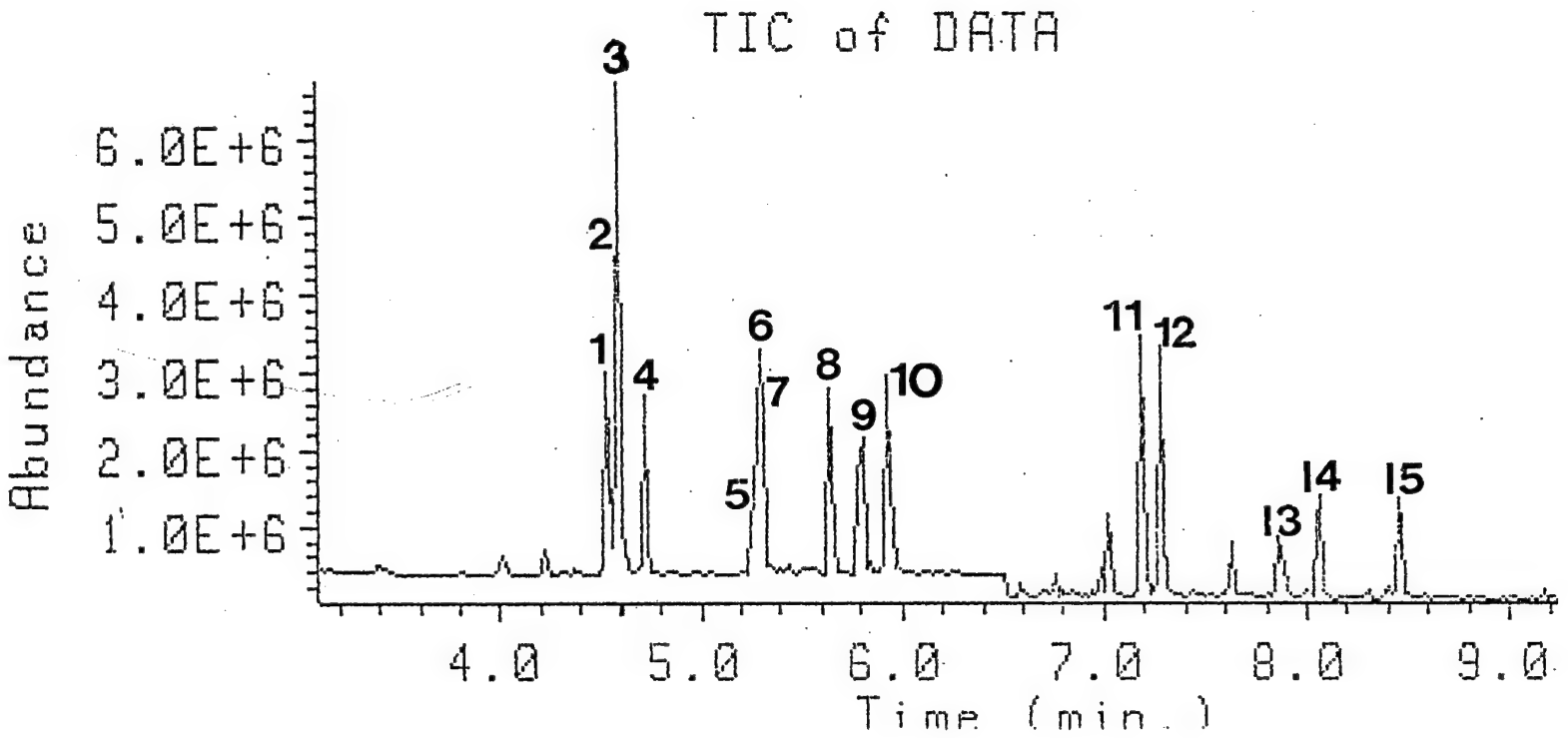


FIGURE 11. SEPARATION OF BNMPA AND METABOLITES FROM OTHER PHENETHYLAMINES
(1=β-phenethylamine; 2=d-amphetamine-d3; 3=d-amphetamine; 4=phentermine; 5=phenylpropanolamine; 6=d,l-methamphetamine-d5; 7=d-methamphetamine; 8=ephedrine; 9=pseudephedrine; 10=phenylephrine; 11=DP2P-OH; 12=DP2P; 13=benzphetamine; 14=N-demethyl-BNMPA;15=BNMPA)

TABLE 3. INTERFERENCE STUDY OF BNMPA AND METABOLITES WITH OTHER PHENETHYLAMINES (HFBA DERIVATIZATION)

COMPOUND	RETENTION TIME (MIN)	IONS MONITORED	COMMENTS
β-PHENETHYLAMINE	4.67	104, 118, 99, 149/150	
AMPHETAMINE-d3	4.72	243, 121, 92	
AMPHETAMINE	4.74	240, 118, 91	
PHENTERMINE	4.85	254, 91	No 210 ion
PHENYLPROPANOLAMINE	5.39	240, 169, 330, 275	
METHAMPHETAMINE-d5	5.42	258, 213, 119	
METHAMPHETAMINE	5.44	254, 118, 210	
EPHEDRINE	5.76	254, 210, 344	No 118 or 91 ions
PSEUDEPHEDRINE	6.06	254, 210, 344	No 118 or 91 ions
PHENYLEPHRINE	6.50	240, 169	No 91 ion
DP2PROPANOL	**7.29**	**194, 91, 193**	**No molecular ion (408)**
DP2P	**7.43**	**91, 119, 210**	**Not derivatized**
BENZPHETAMINE	7.99	148, 91, 65, 149, 92	Not derivatized
N-DEMETH BNMPA	**8.18**	**194, 316, 103**	**No molecular ion (407)**
BNMPA	**8.58**	**330, 133, 194**	**No molecular ion (421)**

TABLE 4. INTERFERENCE STUDY OF BNMPA AND METABOLITES WITH HHS - FUDT ANALYTES (HFBA DERIVATIZATION)

COMPOUND	RETENTION TIME (MIN)	IONS MONITORED	COMMENTS
DP2PROPANOL	**7.22**	**194, 91, 193**	**No molecular ion (408)**
DP2P	**7.33**	**91, 119, 210**	**Not derivatized**
N-DEMETH BNMPA	**8.10**	**194, 316, 103**	**No molecular ion (407)**
BNMPA	**8.50**	**330, 133, 194**	**No molecular ion (421)**
COCAINE	9.76	182, 272, 303	Not derivatized
BENZOYLECGONINE	Not found	485, 364, 380	
PHENCYCLIDINE	8.35	200, 186, 242	Not derivatized
MORPHINE	10.74	464	
CODEINE	10.41	496, 283	
THCA	10.43	495	
6 - MAM	10.74	523, 310	

Chapter 4

Metabolism and Urinary Excretion of α-Benzyl-N-methylphenethylamine (BNMPA), in Humans

4.1 Abstract

The detection of BNMPA or its metabolites in urine samples may provide a marker of use of illicitly synthesized methamphetamine.

One 49-year old, healthy, white male volunteer who admitted to caffeine and nicotine consumption only, ingested 5 mg BNMPA. Seventeen urine specimens were collected over 50 hours post-ingestion. These specimens were analyzed for BNMPA and its four predicted major metabolites by GC/MS following β-glucuronidase hydrolysis or acid hydrolysis, liquid / liquid extraction, and derivatization with HFBA. Specimens were also analyzed without hydrolysis to determine the abundance of non-conjugated ("free") metabolites. Only trace amounts of BNMPA and its N-demethyl metabolites were detected, with maximum excretion from 2 to 4 hours post-ingestion. In the non-hydrolyzed samples, the p-OH metabolites were also present in only trace amounts. Maximum excretion of DP2P was at 2 hours. Following either hydrolysis procedure, p-OH-BNMPA and p-OH-N-demethyl BNMPA were the major metabolites detected. Maximum excretion of these 2 metabolites occurred at 4 hours. With the exception of the parent compound and the N-demethyl

metabolite, excretion of metabolites was greater than the LOD of this procedure (2.5 ng/ml) up to 21 hours post ingestion. Metabolites were detectable in sufficient quantities to serve as an adequate marker of illicit methamphetamine consumption within the preceding 24 hours.

4.2 Introduction

BNMPA is structurally similar to benzphetamine, a Schedule III controlled anorexiant. Common metabolic pathways for most phenethylamine drugs, including benzphetamine, are deamination, N-demethylation, hydroxylation and conjugation (Baselt and Cravey, 1989). Therefore, a logical starting point to elucidating the metabolism of this impurity is to study the metabolism of benzphetamine, summarized in Figure 2 (Inoue and Suzuki, 1986). Based on this scheme, the predicted the major metabolites of BNMPA are the N-demethyl compound, p-OH-BNMPA, p-OH-N-demethyl BNMPA and diphenyl-2-propanone (DP2P) (Figure 3).

This chapter describes the search for these metabolites in human urine following the ingestion of 5 mg of BNMPA by one male volunteer.

4.3 Materials and Methods

4.3.1 Reagents and chemicals

All reagents and chemicals are described in Chapter 2.

4.3.2 Urine samples

One male volunteer ingested 5 mg BNMPA. Seventeen urine specimens were collected over 50 hours post-ingestion. Total urine volume, pH and creatinine were noted for each sample.

4.3.3 Hydrolysis and Derivatization Procedures

All hydrolysis and derivatization procedures are as described in Chapter 2.

4.3.4 Instrumentation

GC/MS analysis was performed as described in Chapter 2.

4.4 Results

Only trace amounts (amounts above the previously determined LOD of 2.5 ng/ml but less than the LOQ of 25 ng/ml) (Chapter 3) of BNMPA and its N-demethyl metabolite were detected, with maximum excretion from 2 to 4 hours post-ingestion. In the non-hydrolyzed samples, the p-OH metabolites were also present in only trace amounts. Maximum excretion of DP2P was at 2 hours (33 ng/ml) in both the hydrolyzed and non-hydrolyzed samples .

p-OH-BNMPA and p-OH-N-demethyl-BNMPA were predicted to be the major metabolites of BNMPA from studies of benzphetamine and other similar compounds (Inoue and Suzuki, 1986). Derivatization, fragmentation and approximate retention times (8.8 minutes for p-OH-N-demethyl-BNMPA and 9.0 minutes for p-OH-BNMPA) were predicted from data obtained from the parent compounds (Chapter 2) as well as benzphetamine/metabolite detection data. We noted much smaller peaks of identical ions and ratios to each predicted p-OH metabolite at 8.7 and 8.9 minutes, respectively, which are believed to be m-OH-BNMPA and m-OH-N-demethyl-BNMPA.

Following β-glucuronidase hydrolysis, p-OH-BNMPA and p-OH-N-demethyl BNMPA were the major metabolites detected, accounting for 4% of the dose over 50 hours (Figure 12). Maximum excretion of these 2 metabolites occurred at 4 hours (Figure 13), with urinary concentrations of 158 ng/ml (0.7 μM)

and 142 ng/ml (0.6 μM), respectively (Table 5). Similar results and quantities were seen following acid hydrolysis (Table 6). With the exception of the parent compound and the N-demethyl metabolite, excretion of metabolites was greater than the LOD of this procedure (2.5 ng/ml) up to 21 hours post ingestion. Total recovery from all metabolites was 4% of the ingested dose.

Since there were only three time points to analyze, we can only approximate urinary excretion rate data. The urinary excretion rate constants (K_m) for phenyl-OH-N-demeth and phenyl-OH BNMPA were -0.18/h and -.21/h, respectively. These were calculated as the slope of the line obtained by plotting the log of the amount excreted per unit time (mean of the collection interval) vs. time (Figure 14 and 15).

4.5 Conclusions

Before beginning the study of the pharmacology/toxicology of any "new" compound, it is necessary to have an understanding of its pharmacokinetics (absorption, distribution, biotransformation and excretion). It is these factors and the amount ingested that ultimately determine the availability of the drug at its site of action and subsequently its overall effect on the organism. Therefore, as a basis for the pharmacological/toxicological study of BNMPA, it was necessary to confirm a predicted metabolic pathway for this compound and ensure that predicted metabolites could be detected in biological fluids.

Based on studies in humans of benzphetamine metabolism, the predicted four major metabolites of BNMPA to be the N-demethyl compound, p-OH-BNMPA, p-OH-N-demethyl BNMPA and diphenyl-2-propanone (DP2P). These four compounds were indeed present in the urine of a human volunteer who had

ingested 5 mg of BNMPA. Additionally, the p-OH metabolites were present at greater than the LOQ of 25 ng/ml at two and four hours post-ingestion and greater than the LOD of 2.5 ng/ml for up to 21 hours post ingestion.

The total recovery of only 4% of the ingested dose was surprisingly low but may be accounted for by several possibilities. While the bioavailability of this compound is unknown, we would predict this lipophilic compound to be well absorbed and have a large apparent volume of distribution (V_d). Second, there are other metabolites which we did not look for. As already stated, it is likely that the small secondary peak in the vicinity of what we are assuming to be the p-OH metabolite peaks may be the m-OH metabolite. Because we do not have standards for confirmation, we did not include this secondary peak in quantitation. Additionally, Inoue and Suzuki (1986) list the methoxy compound as a minor metabolite of benzphetamine. This compound may account for a larger percentage of the metabolites of BNMPA than benzphetamine. Third, in the original method development (Chapter 3), an absolute recovery experiment was not performed using urine controls rather than calibrators. The liquid/liquid extraction method used for these studies using n-butyl chloride as the extraction solvent may not be as efficient as a solid phase extraction method that would take advantage of the polar nature of these compounds. Finally, bile/fecal excretion may be the major route of elimination from the body (Chapter 6).

Glucuronides constitute the majority of Phase-II metabolites of phenols, alcohols and carboxylic acids (Benet and Sheiner, 1985). Minimal amounts of the BNMPA metabolites were excreted unconjugated. Glucuronide appears to be the major conjugate of these metabolites since there was no significant difference in quantities recovered from acid hydrolysis vs. β-glucuronidase hydrolysis.

The p-OH-N-demethyl-BNMPA metabolite was detectable in sufficient quantities to serve as an adequate marker of illicit methamphetamine consumption within the preceding 24 hours.

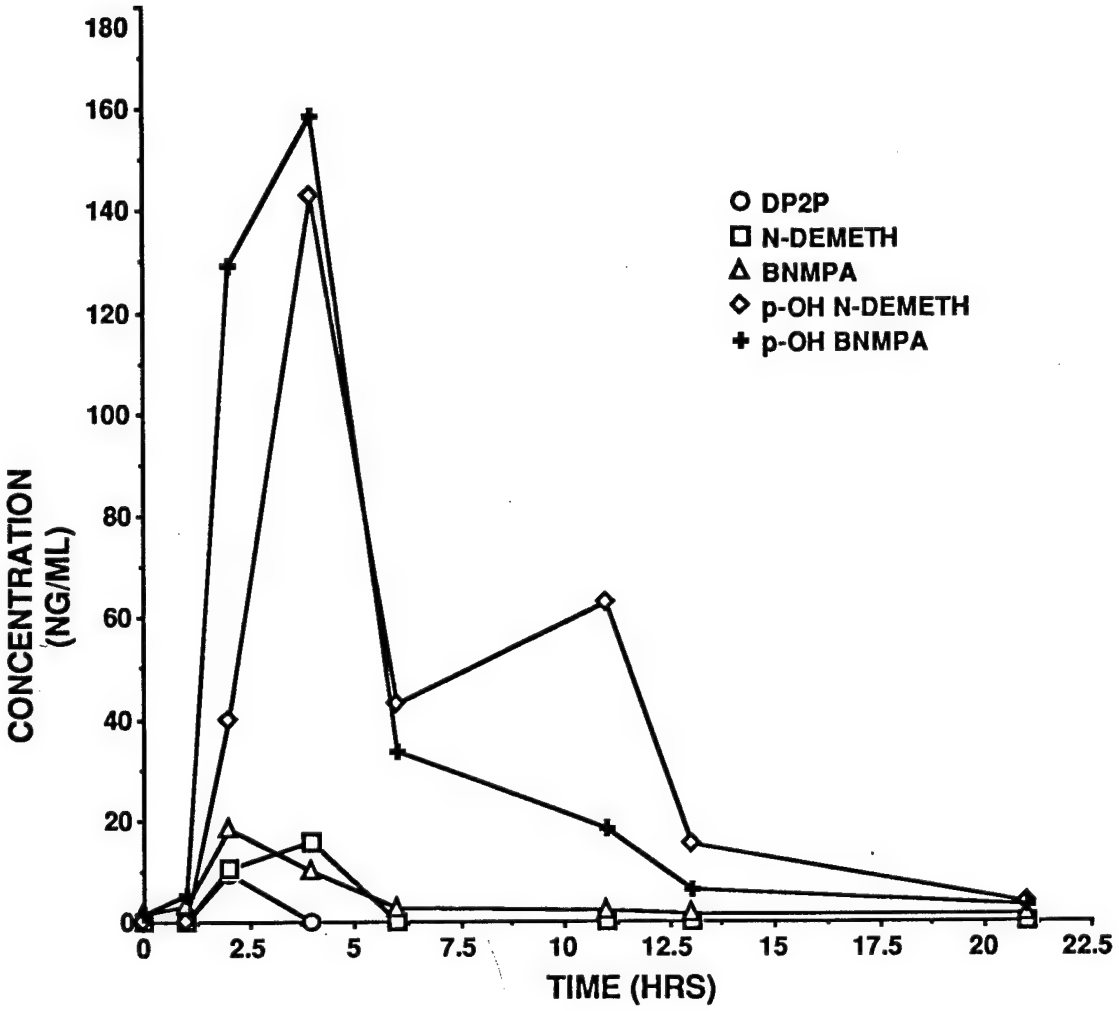


FIGURE 12. EXCRETION PLOT OF BNMPA AND METABOLITES

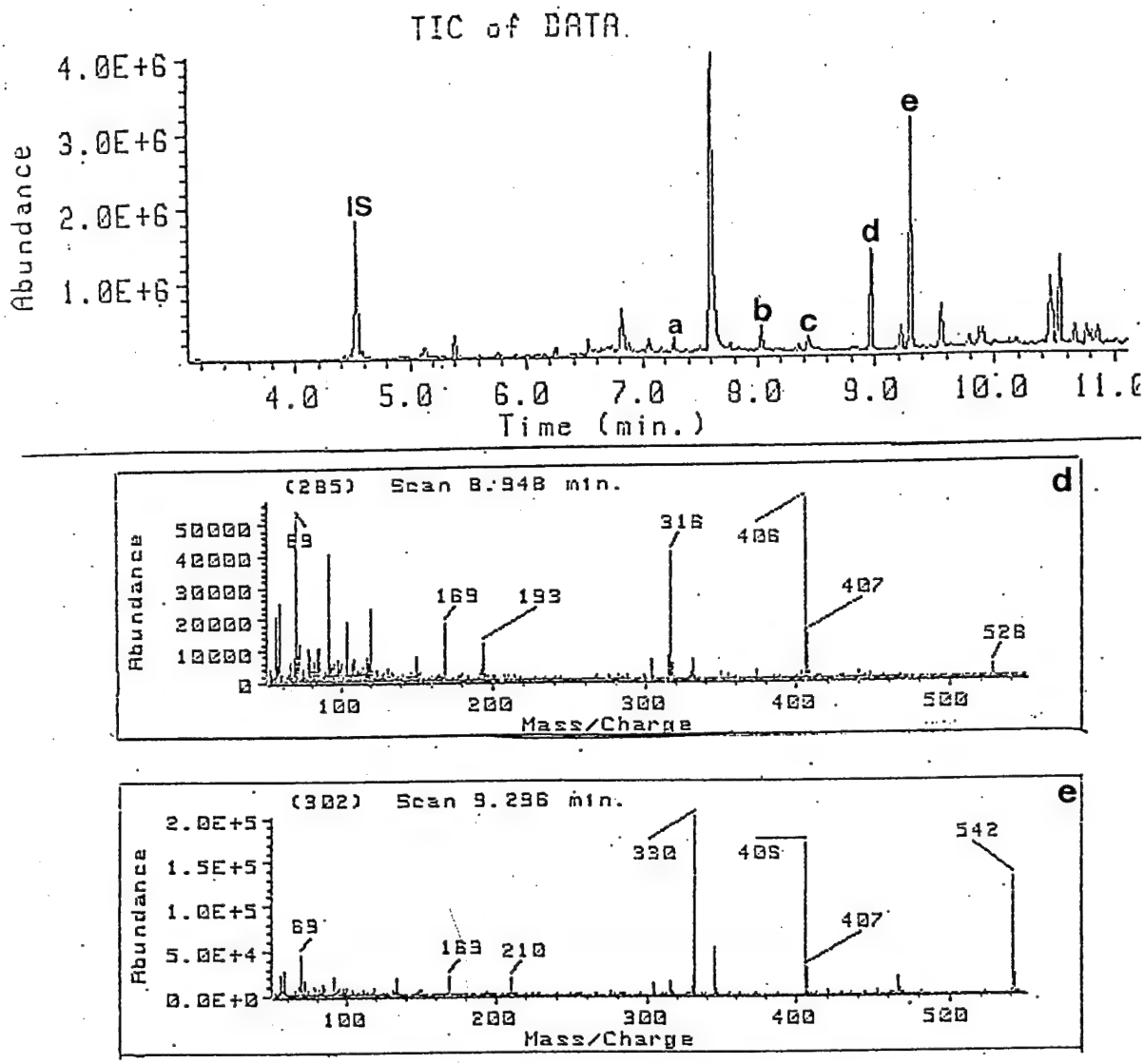


FIGURE 13. TOTAL ION CHROMATOGRAM OF 4-HOUR URINE SAMPLE
(IS=Internal Standard; a=DP2P; b=N-demethyl-BNMPA;
c=BNMPA; d=p-OH-N-demethyl-BNMPA; e=p-OH-BNMPA)

TABLE 5. p-OH METABOLITES EXCRETION DATA

TIME (HRS.)	URINE VOLUME (MLS)	p-OH-N-demethyl-BNMPA		p-OH - BNMPA	
		AMOUNT DETECTED (ng / ml)	TOTAL EXCRETED (ug)	AMOUNT DETECTED (ng / ml)	TOTAL EXCRETED (ug)
0	50	0	0	0	0
1	325	0	0	0	0
2	320	40	12.7	130	41.4
4	310	142	44.2	158	49.1
6	355	43	15.2	33	11.8
11	240	63	15.0	0	0
13	200	0	0	0	0
21	625	0	0	0	0

TABLE 6. ACID vs. β-GLUCURONIDASE HYDROLYSIS DATA

TIME (HRS.)	URINE VOLUME (MLS)	p-OH-N-demethyl-BNMPA AMOUNT DETECTED (ng / ml)		p-OH - BNMPA AMOUNT DETECTED (ng / ml)	
		Acid Hydrolysis	β-glucuronidase	Acid Hydrolysis	β-glucuronidase
0	50	0	0	0	0
1	325	0	0	0	0
2	320	61	40	116	130
4	310	95	142	154	158
6	355	63	43	49	33
11	240	29	63	0	0
13	200	0	0	0	0
21	625	0	0	0	0

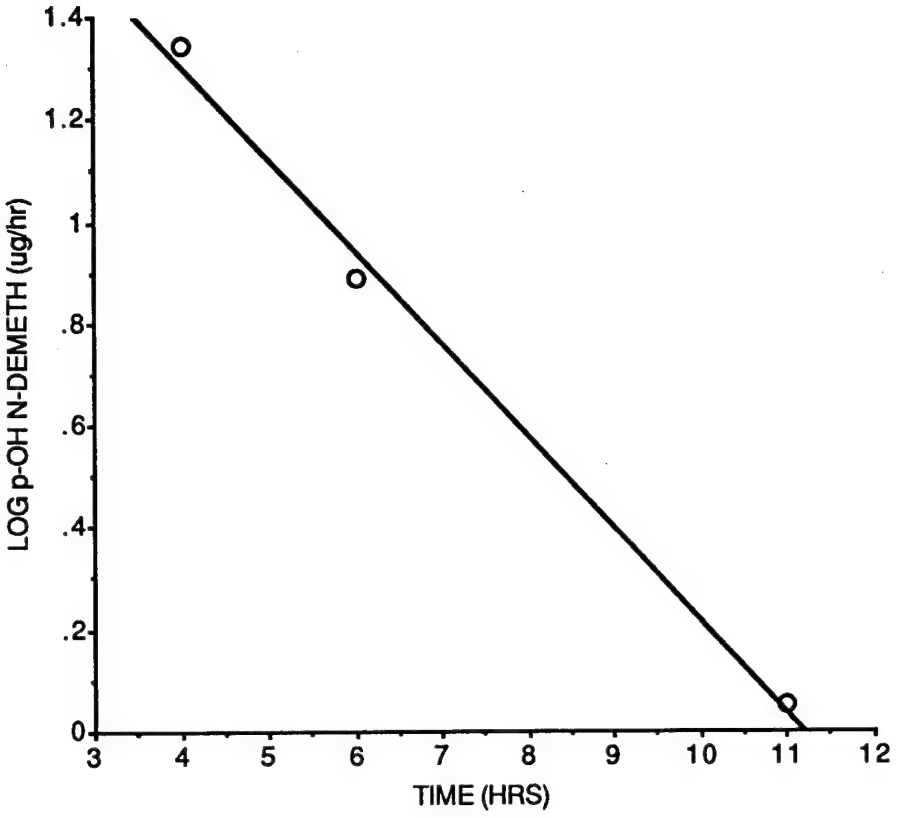


FIGURE 14. URINARY EXCRETION RATE CONSTANT OF p-OH-N-DEMETH-BNMPA
($y = -0.18x + 2.0281$, $r^2 = .9951$, $K_m = -0.18/h$)

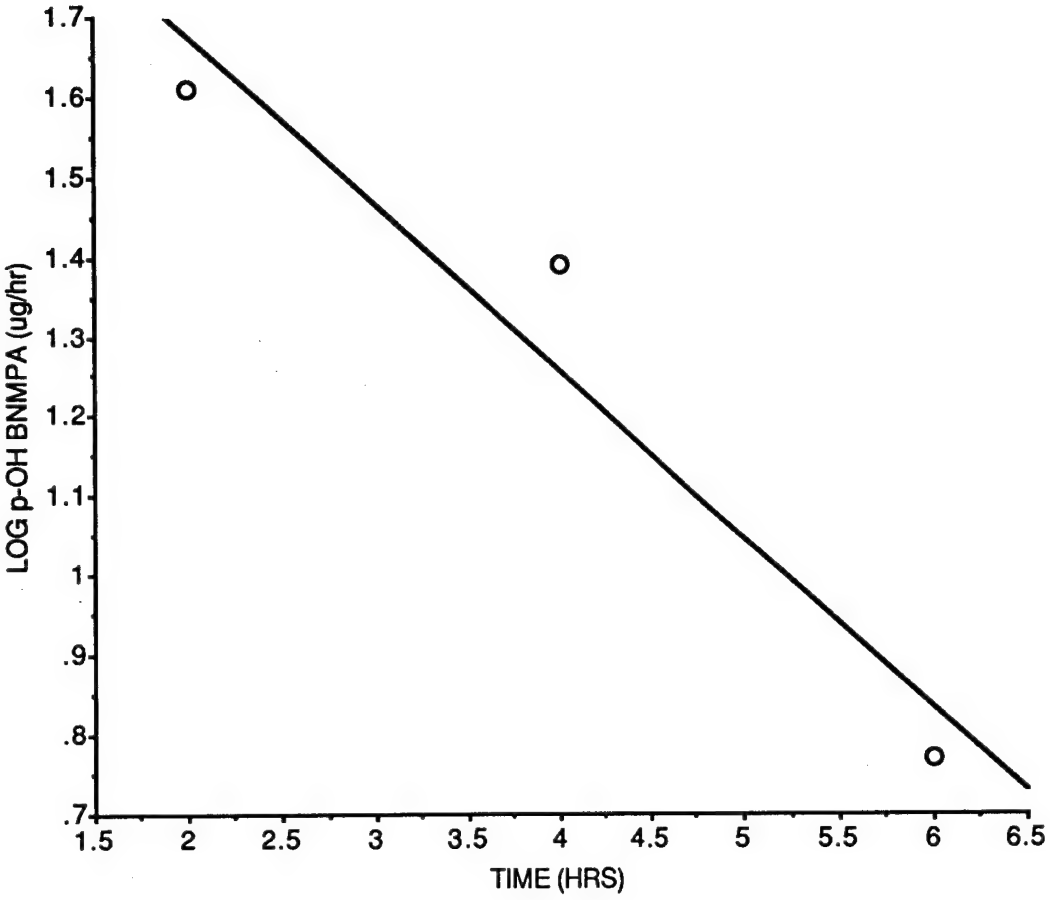


FIGURE 15 URINARY EXCRETION RATE CONSTANT OF BNMPA
($y = -.21x + 2.0967$, $r^2 = .9297$, $K_m = -.21/h.$)

Chapter 5

Detection of BNMPA and Metabolites in Urine of Methamphetamine Users

5.1 Abstract

The detection of BNMPA or its metabolites in urine samples may provide a marker of use of illicitly synthesized methamphetamine. We have previously demonstrated the four major metabolites of BNMPA to be the N-demethyl compound, diphenyl-2-propanone (DP2P), p-hydroxy-N-demethyl BNMPA, and p-hydroxy-BNMPA. Two-hundred urine samples which had screened / confirmed methamphetamine / amphetamine positive were obtained from a large west coast urine drug testing laboratory doing analyses for emergency rooms, drug rehabilitation programs and the criminal justice system. These specimens were analyzed for BNMPA and its four predicted major metabolites by gas chromatography / mass spectrometry following β-glucuronidase hydrolysis, liquid / liquid extraction, and derivatization with heptafluorobutyric anhydride (HFBA). Two samples contained detectable amounts of BNMPA and / or its metabolites. One of these contained trace amounts (greater than the limit of detection but less than the limit of quantitation)of N-demethyl BNMPA and DP2P as well as 36 ng/ml p-OH-N-demethyl-BNMPA. The other sample contained trace amounts of BNMPA, p-OH-BNMPA and p-OH-N-demethyl-BNMPA as well as 28 ng/ml N-demethyl-BNMPA. Metabolites were detectable in sufficient quantities to serve as

an adequate marker of illicit methamphetamine consumption within the preceding 24 hours.

5.2 Introduction

Since impurities arising from the illicit synthesis of drugs can be characteristic of a particular synthetic method, their presence in seized samples or their detection in biological samples from users can further be used to monitor the sales of precursor chemicals, to group seized compounds to common sources of illicit production or provide links between manufacturers, dealers and users.

This communication presents the results of the analysis for BNMPA and its metabolites in eighty urine samples which had been screened/confirmed "positive" for methamphetamine/amphetamine at a large west coast drug testing laboratory. Prior to screening these urines, p-OH-BNMPA was synthesized to confirm the prediction of the retention time and GC/MS fragmentation pattern of this major metabolite.

5.3 Materials and methods

5.3.1 Urine samples

Urine samples were obtained from a large west coast drug testing laboratory performing analyses for emergency rooms, drug rehabilitation programs and the criminal justice system. Only samples which had screened "positive" for methamphetamine/amphetamine were analyzed.

Forty of these samples were received in December, 1993 and represented samples which had been received throughout 1993 and had been confirmed

methamphetamine "positive". The second group of 160 samples were received in September, 1994 and represented samples which only screened methamphetamine/amphetamine "positive" from the 2-3 months immediately preceding their shipment.

5.3.2 Hydrolysis and derivatization

β-Glucuronidase hydrolysis, extraction and HFBA derivatization were carried out as described in Chapter 2.

5.3.3 Instrumentation

GC/MS analysis was performed on a Hewlett-Packard 5890 GC equipped with a 12 m. x 0.2 mm(id) x 0.33 μm (film thickness) HP-1 capillary column connected to a Hewlett-Packard 5971-A mass selective detector, as described in Chapter 2.

5.4 Results

Two groups of urine samples were received from the west coast drug testing laboratory. The first group, received in December, 1993, contained forty samples which had been screened in their laboratory using Emit®–d.a.u. Amphetamine Class Assay (EC) (Syva Company, San Jose, CA). They confirmed and quantitated methamphetamine / amphetamine in these samples by GC/MS. All of these samples were analyzed for BNMPA / metabolites. Two samples from this group of forty contained detectable amounts of BNMPA and / or its metabolites. One of these contained trace amounts (greater than the limit of detection of 2.5 ng/ml but less than the limit of quantitation of 25 ng/ml) of N-demethyl BNMPA and DP2P as well as 0.04 mg/L (0.2 μM) p-OH-N-demethyl BNMPA. This sample contained 45 mg/L (302 μM) methamphetamine and 1.2

mg/L (8.8 μM) amphetamine. The other sample contained trace amounts of BNMPA, p-OH-BNMPA and p-OH-N-demethyl-BNMPA as well as 0.03 mg/L (0.1 μM) N-demethyl BNMPA. The methamphetamine and amphetamine amounts were 25 mg/L (168 μM) and 3.1 mg/L (23 μM), respectively.

The second group, received in September, 1994, contained 160 samples which had only been screened as "positive" for methamphetamine/amphetamine in their lab using the EC assay. We further screened these samples in our laboratory using the Emit®-II Amphetamine/Methamphetamine Assay (EM). Eighty-eight of the 160 samples were "positive" using this assay. These 88 samples were analyzed for BNMPA / metabolites. Of these, 40 were confirmed methamphetamine / amphetamine positive. None of these samples, representing samples collected at least one year later than the original group, contained BNMPA or its metabolites.

METH and AMPH concentrations as well as other compounds detected in these 80 confirmed methamphetamine-positive samples are summarized in Table 7.

5.4 Conclusions

Recently, difficulties in obtaining P2P and other precursors necessary for the reductive amination methods of methamphetamine synthesis, combined with the increased availability of (-)-ephedrine and (+)-pseudephedrine both in this country and the Far East, has resulted in the conversion of clandestine synthesis of METH to the ephedrine reduction methods (*Microgram,* 1994). We believe this change in the illicit synthesis of METH may account for the fact that 2 out of the 40 "older" samples we analyzed contained BNMPA and its metabolites. In

contrast, none of the samples that had been collected since mid-1994 contained the impurity resulting from reductive amination.

BNMPA and its metabolites are present in sufficient quantities to serve as a markers of illicit consumption (within the previous 24 hours) of methamphetamine synthesized with reductive amination methods using P2P synthesized from phenylacetic acid.

TABLE 7. METH AND AMPH CONCENTRATIONS AND OTHER COMPOUNDS DETECTED IN URINE SAMPLES SCREENED "POSITIVE" FOR "AMPH / METH" BY Emit®- II ([a] = mg/L)

No	METH CONC [a] (GC/MS)	AMPH CONC [a] (GC/MS)	OTHER CPDS. DETECTED
1	1.2	0.7	
2	7.8	1.2	BNMPA METABS
3	11.9	2.4	
4	1.5	0.6	
5	60.0	6.6	
6	0.3	0.6	
7	5.2	0.9	
8	5.9	0.8	
9	77.3	8.5	
10	4.6	1.0	
11	12.2	1.3	
12	1.4	0.6	
13	0.7	0.2	
14	0.4	0.6	
15	2.1	2.7	
16	0.6	0.4	
17	0.2	0.2	
18	35.0	7.5	
19	0.6	0.4	
20	> 50.0	13.5	
21	3.1	1.0	
22	2.1	0.7	
23	25.0	3.1	BNMPA METABS
24	1.8	0.03	
25	2.2	0.8	
26	16.2	6.2	
27	7.6	1.7	
28	11.8	2.9	
29	5.9	2.2	
30	0.5	0.2	
31	4.4	7.9	
32	1.1	3.3	
33	0.4	0.4	
34	0.9	0.3	
35	1.3	0.2	
36	0.9	0.3	
37	30.0	7.6	
38	11.3	2.3	
39	0.1	0.3	
40	1.4	0.3	

No	METH CONC [a] (GC/MS)	AMPH CONC [a] (GC/MS)	OTHER CPDS. DETECTED
41	26.1	3.3	
42	10.7	1.5	
43	6.3	0.2	
44	1.6	3.2	
45	0	2.7	
46	5.6	0.3	
47	0.3	0.1	
48	0	0	PHENOTHIAZINE
49	0	0	EPHEDRINE
50	0	0	PHENOTHIAZINE
51	32.0	0.2	p - OH - BNMPA
52	0.3	0.2	
53	18.3	> 5.0	
54	0.1	0.2	
55	14.4	0.8	
56	> 5.0	0.3	
57	> 5.0	2.2	
58	> 5.0	0.4	
59	0.8	0.1	
60	> 5.0	4.1	
61	0.4	0.2	
62	0.7	0.5	
63	0	> 5.0	
64	0	0	EPHEDRINE
65	> 5.0	0.7	PSEUDEPH
66	0	0	EPHEDRINE
67	0	0	PPA
68	0.8	0.3	
69	0.8	0.1	PSEUDEPH
70	0	0	PHENTERMINE
71	0	0	EPHEDRINE
72	0.4	0.3	
73	0.6	0.2	
74	2.2	1.0	
75	0.08	0.4	
76	> 5.0	0.2	
77	0.8	0.8	
78	0	0	EPHEDRINE
79	1.2	1.2	
80	7.3	0.4	EPHEDRINE

Chapter 6

The Detection of a Metabolite of α-Benzyl-N-methylphenethylamine (BNMPA), in a Mixed Drug Fatality Involving Methamphetamine

6.1 Abstract

A 37-year old, white male collapsed at his home following a party. He reportedly had a history of unspecified cardiac arrhythmia. The ambulance crew found him unresponsive and an ECG revealed ventricular tachycardia / fibrillation. Following one hour of resuscitative efforts in the ambulance and emergency room of a local hospital, he was pronounced dead. A urine toxicology screen at the hospital was "positive" for benzodiazepines, cocaine and amphetamine/methamphetamine. At autopsy, there was generalized organ congestion with no evidence of trauma or other significant pathology except mild, left ventricular hypertrophy. Quantitation by gas chromatography/mass spectrometry (GC/MS) of methamphetamine in bile, blood, urine and gastric contents yielded 21.7, 0.7, 32.0 and 2.9 mg/L, respectively. Liver and brain contained 2.2 and 2.7 mg/kg, respectively. A trace amount of p-OH-α-benzyl-N-methylphenethylamine (p-OH-BNMPA), a metabolite of α-benzyl-N-methylphenethylamine (BNMPA), an impurity of illicit methamphetamine synthesis was also detected in the urine. Since these impurities can be characteristic of a particular synthetic method, their presence in seized samples or their detection in biological samples from methamphetamine users can further

be used to monitor the sales of precursor chemicals, to group seized compounds to common sources of illicit production or provide links between manufacturers, dealers and users.

6.2 Introduction

Methamphetamine ("speed") continues to rank among the top twenty drugs in emergency room and medical examiner mentions (DAWN). However, in 1993, drug abuse deaths involving methamphetamine accounted for only 0.5% of total drug abuse deaths in 14 large metropolitan areas (including Washington D.C. and Norfolk, Virginia) east of the Mississippi river compared to 12 % of total drug abuse deaths in 11 large metropolitan areas west of the Mississippi river.

Methamphetamine is readily synthesized in clandestine laboratories. Illicitly obtained methamphetamine is frequently impure, containing various purposefully added diluents and adulterants, as well as impurities of manufacture and origin. One such impurity is α-benzyl-N-methylphenethylamine (BNMPA). We have previously demonstrated the four major metabolites of BNMPA to be the N-demethyl compound, diphenyl-2-propanone (DP2P), p-hydroxy-N-demethyl BNMPA, and p-hydroxy-BNMPA. We have also shown that these metabolites are detectable in known methamphetamine users in sufficient quantities to provide a marker of use of illicitly synthesized methamphetamine.

Because of its rare occurrence in east coast medical examiners cases and because of our interest in the detection of impurities of illicit methamphetamine manufacture in biological samples as a means of tracking supply sources, we present a case of a "mixed drug" fatality involving methamphetamine.

6.3 History and autopsy results

A 37-year old, white male collapsed at his home at approximately 3:00 a.m. following a party. The ambulance arrived at approximately 4:30 a.m. where an ECG revealed ventricular tachycardia / fibrillation. Resuscitative efforts were initiated and the patient was transported to the emergency room at a major local teaching hospital where he was pronounced dead at 5:05 a.m. An antemortem urine toxicology screen performed at the hospital was "positive" for amphetamine, benzodiazepines and cocaine. Family and friends reported a history of unspecified cardiac arrhythmia or "other cardiac problem".

At autopsy there was generalized organ congestion but no evidence of trauma or other significant pathology apart from cardiomegaly (510 grams). There was mild left ventricular hypertrophy. Postmortem toxicology revealed blood concentrations of 0.21 mg/L benzoylecgonine, 0.09 mg/L morphine, 0.68 mg/L methamphetamine and 0.13 mg/L diazepam. No ethanol, methanol, isopropanol or acetone was detected. The death was classified as an accidental, mixed drug toxicity.

6.4 Materials and methods

Quantitation of methamphetamine and BNMPA and metabolites in blood, urine, bile liver, brain and gastric samples was by gas chromatography / mass spectrometry (GC/MS). Blank tissue samples were drug-free autopsy specimens obtained from a major local teaching hospital. Tissue samples were homogenized and suspended in twice their volume of normal saline. Bile samples were diluted 1:3 with normal saline. A total of 4 ml. of homogenate, diluted bile, urine or blood was used for blanks, calibrators and samples. β-glucuronidase

hydrolysis, extraction, derivatization and GC/MS conditions were as described in Chapter 2.

6.5 Results

Bile, blood, urine and gastric contents contained 21.7, 0.7, 32.0 and 2.9 mg/L methamphetamine, respectively. Liver and brain contained 2.2 and 2.7 mg/kg, respectively. A trace amount of p-OH-BNMPA was also detected in the urine.

6.6 Conclusions

As is true with most abused drugs, the phenomena of tolerance makes blood and tissue levels as predictors of cause or contribution to death very difficult (Karch, 1993). This variability in blood and tissue concentrations of methamphetamine in fatalities can be seen in the cases summarized in Table 8. Methamphetamine concentrations in fatal cases have ranged from less than 1 mg/L to over 14 mg/L (Cravey and Reed, 1970; Kojima, et al., 1984; Matoba, et al., 1985; Baselt and Cravey, 1989). Since the degree of tolerance for any drug is impossible to determine at autopsy, attributing significance to isolated amounts is unwise (Karch, 1993). Low levels, thought to be incidental findings, are hard to interpret. As in the case presented here, very low concentrations (0.7 mg/L) have been observed in patients dying of what is now described as "classic stimulant toxicity" with agitation, hypertension, tachycardia and hyperthermia (Fukunaga, et al., 1987).

It is also intriguing to note the presence of significant quantities of methamphetamine/amphetamine in the bile from this case. It is well established

TABLE 8. METHAMPHETAMINE (METH) AND AMPHETAMINE (AMPH) TISSUE CONCENTRATIONS IN OVERDOSE FATALITIES INVOLVING METHAMPHETAMINE

REFERENCE	N	BLOOD[a]		BRAIN[b]		GASTRIC		LIVER[b]		URINE[a]	
		Meth	Amph	Meth	Amph	Meth	Amph	Meth	Amph	Meth	Amph
Zalis et al.(1963)	1	1.3	X	X	X	X	X	X	X	X	X
Cravey et al. (1970)	1	40	X	X	X	X	X	X	206	X	X
Baselt et al. (1995)	1	4.3	X	X	X	X	X	X	X	28	X
"	1	2.0	X	X	X	1.5 mg	X	4.8	X	28	X
Kojima et al. (1984)	1	5.6	0.35	102	0.8	11.6 mg	0.2 mg	175	1.3	320	10.0
Bailey et al. (1989) Meth-related deaths (AO)	11	0.02-3.05	NMA - 0.32	X	X	X	X	0.17 - 11.6	NMA - 1.06	X	X
Bailey et al. (1989) Mixed cocaine / meth deaths (AO)	6	0.08 - 1.01	0.03 - 0.17	2.99 (N=1)	0.4 (N=1)	X	X	0.45 - 1.29	0.11 - 0.73	X	X
Katsumata et al. (1993)	1	9.0	0.2	22	X	51 mg/L	X	45	X	103	0.53

[a] = mg / L; [b] = mg / kg; X = not done; NMA = No measurable amount; AO = Accidental overdoses

that bile may be an important route of excretion for many drugs and that enterohepatic circulation of drugs may contribute to their total pharmacokinetic profile (Caldwell, 1976). The biliary excretion of many amphetamines has been studied in the rat with 15 to 20% of the dose excreted in the bile with enterohepatic circulation. However, the molecular weight requirement for significant biliary excretion varies with species, being 325 ± 50 in the rat. In humans, the cut-off is not so clear, but it has been thought that only compounds with molecular weight of 500 to 1000 are excreted significantly in human bile. Since virtually all amphetamines and metabolites have molecular weights less than 375, it has not been expected that biliary excretion is an important route for these compounds in man. In fact, bile concentrations of methamphetamine/amphetamine are rarely reported in the literature. Only Di Maio and Garriott (1977) reported a bile concentration of 135 mg/L in an amphetamine ingestion overdose fatality. That report, coupled with the finding of 21.7 mg/L methamphetamine and 0.58 mg/L amphetamine in the bile in the case presented here, suggests that bile may be a useful tissue for the detection of these compounds in suspected methamphetamine fatalities.

Studies on the toxicity of BNMPA are preliminary and have yet to provide definitive answers to the question of BNMPA's effects in vivo. The possibility that impurities of manufacture may be contributing to the toxicity of METH in chronic abusers, especially in "low-concentration" fatalities such as we describe here, cannot be eliminated

Finally, impurities arising from the illicit synthesis of methamphetamine can be characteristic of a particular synthetic method. Therefore, their presence in seized samples or their detection in biological samples from methamphetamine

users can further be used to monitor the sales of precursor chemicals, to group seized compounds to common sources of illicit production or provide links between manufacturers, dealers and users. Because the P2P/Leukart method is currently unpopular, the finding of a metabolite of an impurity arising from this method in this case is unique enough to provide conclusive evidence that the methamphetamine involved in this case was illicitly manufactured. Since the DEA monitors synthetic methods used by various labs, this impurity may also provide a link from user to distributor to manufacturer.

Chapter 7

Pharmacological and Toxicological Evaluation of α-benzyl-N-methylphenethylamine (BNMPA), and Interaction with Methamphetamine in Mice

7.1 Abstract

Acute toxicity and spontaneous activity (locomotor) studies were conducted with BNMPA alone and in combination with S(+)-methamphetamine (METH) in male, ICR mice . In the acute toxicity studies, the mice were scored from 0-4 based on whether or not convulsions had to be induced by handling and the type of convulsion (tonic vs. tonic-clonic). While BNMPA produced some behavioral disturbances similar to those seen with methamphetamine (stereotopy, etc.) at doses greater than 30 mg/kg, no true convulsions which fit our criteria were noted until pre-terminal convulsions at 50 mg/kg. The CD_{50} and LD_{50} for this study were 50 mg/kg and 70 mg/kg, respectively. When BNMPA was given in combination with METH, there was no significant difference in the convulsion profile from that of METH given alone. In spontaneous activity studies, doses of BNMPA ranging from 1 mg/kg to 50 mg/kg failed to alter locomotor activity significantly from controls. Spontaneous activity was also measured when BNMPA (5 mg/kg) was combined with METH (5 mg/kg). Spontaneous activity with this combination was not significantly different from 5 mg/kg METH

given alone. We concluded there is no significant interaction between BNMPA and METH.

7.2 Introduction

When one considers the apparent increase in emergency room visits and medical examiner cases since illicit manufacture became the primary source of methamphetamine and the possibility of ingestion of large quantities of impurities, it is surprising that the information concerning the pharmacology of the impurities of manufacture is very limited. However, even preliminary studies indicate that the α-benzyl compounds with CD_{50}'s much less than their LD_{50}'s cause greater CNS stimulation at the brainstem and cord levels than that seen with amphetamine/methamphetamine. This certainly points out the potential danger of street drugs containing substantial amounts of these impurities.

The purpose of this study is to further evaluate the pharmacology and toxicology of BNMPA alone and with METH. It is important to consider the interaction of BNMPA with METH since, as an impurity of METH manufacture, BNMPA is ingested with METH and would rarely, if ever, be consumed by itself. While the classic mechanisms of drug interactions were considered (variously described as "synergy", "additivity", "superadditivity", etc."), the toxicity of BNMPA must also be broadly defined to include antagonism of the effects of METH. If BNMPA significantly depressed the activity produced by METH, the METH user would ingest more of the METH to reach the feeling he /she was used to until its toxic level was approached.

7.3 Materials and Methods

7.3.1 Animals

Male ICR mice, weighing 18-20 grams on delivery, were purchased from Harlan (Dublin, Virginia). Water and food (Rodent Laboratory Chow, Ralston-Purina Co., St. Louis, MO) were available *ad libitum* in both the test and home cages. Groups of 5 were housed in standard mouse cages (18 x 29 x 13 cm) with wood chip bedding in a controlled temperature room (22-24° C) with a12-hr light-dark cycle.

7.3.2 Spontaneous activity studies

Spontaneous activity was measured as the number of interruptions of 16 photocells/cage in an "Omnitech Spontaneous Activity System" (Omnitech Electronics, Columbus, Ohio). The locomotor arena is a12.5 cm x 20 cm x 33.5 cm clear plastic cage with a wire mesh top. Results were compiled using "Digiscan, v. 2.2" software (Omnitech Electronics, Columbus, Ohio).

In an initial experiment, mice were moved to the test room on the day before testing for acclimation. On the test day, subjects were placed individually in the activity chambers for an adaptation period of 30 minutes. After the adaptation period, they were removed from the chambers and given an intraperitoneal (ip) injection of either saline, 1, 3, 5 or 10 mg/kg (0, 7.4, 25, 38 or 74 μM) BNMPA, 5 mg/kg (57 μM) METH or 5mg/kg METH combined with 5 mg/kg BNMPA. Spontaneous activity counts were taken in 10 minute bins for a total of 30 minutes. There were 12 animals in each dosage group.

A second experiment was conducted to examine the effects of BNMPA under different conditions and to compare its activity to another stimulant (AMPH). Four groups of 6 mice each were established and brought to the testing

room the day before testing. Following a 10 minute adaptation period in the test chamber, the mice were given ip injections of either saline, 10 or 50 mg/kg (0, 7.4 or 369 μM) BNMPA, or 5 mg/kg (62 μM) AMPH. Spontaneous activity counts were taken in 10 minute bins for a total of 40 minutes.

7.3.3 Acute toxicity studies

The primary interest in this study was the interaction of BNMPA and METH. However, prior to looking at the interaction of these compounds, it was necessary to establish a baseline effect for each compound individually under the same operating conditions which the interaction studies would be conducted. The purpose of the initial study was to establish a dose-response relationship for BNMPA and METH alone. The observer was blind to all drug conditions.

On the day before testing, all mice were moved to the test room for adaptation. On the test day, each of the 10 mice in a group were marked and weighed. Subjects were administered ip injections of either saline, BNMPA (0,10, 30, 50, 70 or 80 mg/kg; 0, 74, 221, 369, 518, 592 μM) or METH (0,10, 30, 50 or 70 mg/kg; 0, 114, 342, 570, 798 μM). There were10 mice in each dosage group. Immediately following the injections, the observer lifted each mouse gently by the tail, observed quickly for the appearance of tonic or clonic convulsions, then gave a slow 180º turn and observed again for convulsions. Scores were given as follows (Evans and Balster, 1993):

0 = no effect in either observation

1 = tonic convulsion when mouse is lifted and turned

2 = tonic convulsion when mouse is lifted and not turned

OR tonic-clonic convulsion when mouse is lifted and turned

3 = tonic-clonic convulsion when mouse is merely lifted

4 = tonic-clonic convulsion observable either before mouse is lifted or after release

The purpose of the second study was to evaluate whether BNMPA would alter the effects of METH. All conditions were identical to the above protocol except the injections. Subjects received either saline, 1, 3, 5 or 10 mg/kg (11, 38, 57 or 114 μM) METH alone or one of those doses combined with 10 mg/kg (74 μM) BNMPA.

7.3.4 Statistical analysis

Total activity counts were analyzed using ANOVA, and the Newman-Keuls test was used for post-hoc analysis when appropriate. Differences were considered significant at the $p < 0.05$ level.

The Kruskal-Wallis One-Way Analysis of Variance was used to analyze the categorical data in the acute toxicity studies. The dose which caused 50 % of the mice to exhibit seizure activity (CD_{50}) was calculated using the method of Litchfield and Wilcoxon (1949).

7.4 Results

7.4.1 Spontaneous activity

The Interaction Bar Plot for the means of the "Total Counts" for experiments 1 and 2 are shown in Figures 16 and 17, respectively. Figure 16 depicts the mean total activity counts for the thirty minute observation period plotted vs. dosages of BNMPA alone and in combination with METH. A significant effect of treatment with METH was found when compared to controls and all doses of BNMPA alone [$F(6,77) = 34.3$, $p < 0.05$]. METH combined with BNMPA exhibited significantly more activity than each of the other treatments

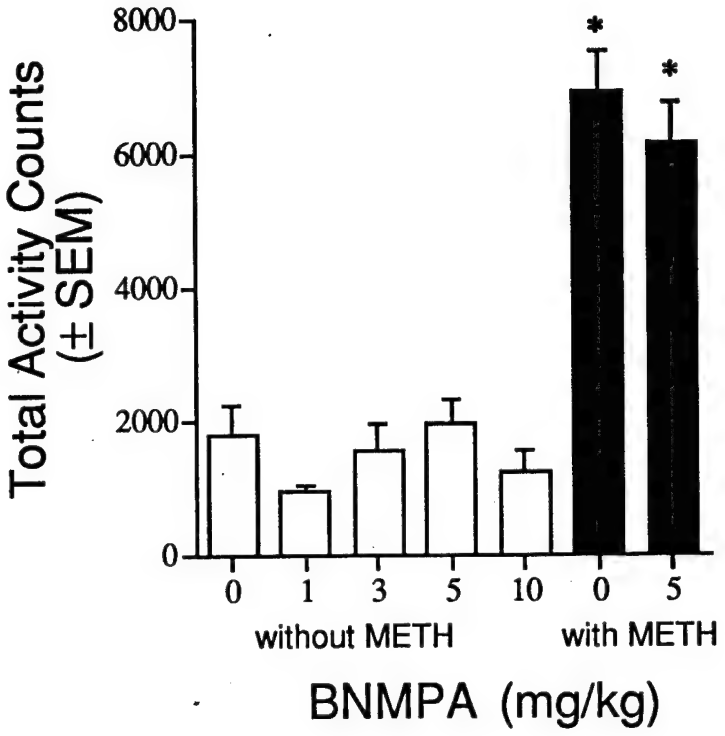


FIGURE 16. THE LOCOMOTOR EFFECTS (MEAN ACTIVITY COUNTS + SEM) OF SALINE, BNMPA (1,3,5 OR 10 mg/kg) AND METH (5 mg/kg) ALONE AND COMBINED WITH 5 mg/kg BNMPA; n=12.
(* = SIGNIFICANTLY DIFFERENT FROM SALINE AND EACH OF THE DOSES OF BNMPA; SIGNIFICANCE LEVEL = 5%)

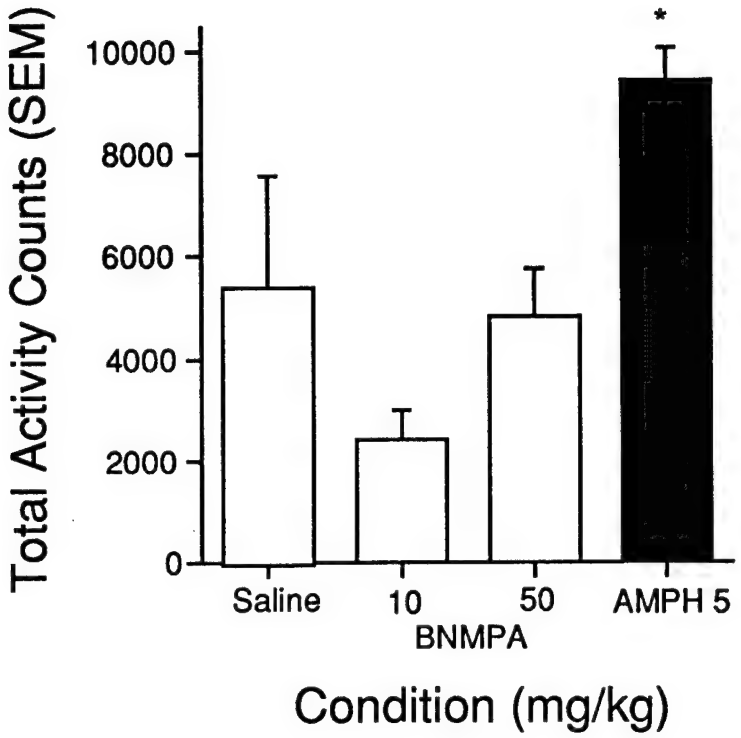


FIGURE 17. THE LOCOMOTOR EFFECTS (MEAN ACTIVITY COUNTS + SEM) OF SALINE, BNMPA (10 OR 50 mg/kg) AND AMPH (5 mg/kg); n=6.
(* = SIGNIFICANTLY DIFFERENT FROM SALINE AND EACH OF THE DOSES OF BNMPA; SIGNIFICANCE LEVEL = 5%)

($p < 0.05$) except METH alone. There were no significant differences in the first 10-minute time bin.

The mean total activity counts for the forty minute observation period plotted vs. 10 and 50 mg/kg doses of BNMPA and 5mg/kg AMPH alone is shown in Figure 17. ANOVA reveals a significant effect [$F(3,20) = 5.3$, $p<0.05$]. The group that received AMPH alone differed significantly from all other groups ($p<0.05$). No other significant differences were found. There were no significant differences in the first 10-minute time bin.

7.4.2 Acute toxicity study

Results are compiled for BNMPA, METH and BNMPA combined with METH in Tables 9-11, respectively. BNMPA as well as METH elicited stereotopy, catatonic-like states and "popcorn-like" hyperactivity beginning at 30 mg/kg (221 μM) and 10 mg/kg (114 μM), respectively. However, because this behavior did not meet the convulsion criteria, these animals were scored as "0" in this study. All of the animals that exhibited spontaneous clonic-tonic convulsions at any point during or throughout the one hour observation period of this study were scored as "4" whether or not they recovered after one-hour. The number of animals that died following tonic-clonic convulsions during the observation period are indicated in each table.

Spontaneous tonic-clonic convulsions were observable with BNMPA at doses lower than those causing lethality [CD_{50} = 41 mg/kg (303 μM); confidence limits = 33-50; $X^2(5)=40.8$, $p<0.05$]. A significant effect of convulsions was found with METH [$X^2(4)=14.1$, $p< 0.05$] but not until reaching a lethal dose. One animal in the 50 mg/kg METH alone group died acutely following injection without exhibiting convulsions.

TABLE 9. CONVULSANT BEHAVIOR IN MICE FOLLOWING INTRAPERITONEAL INJECTIONS OF BNMPA. NUMBERS ARE NUMBER OF ANIMALS OUT OF A GROUP OF 10 THAT EXHIBITED EACH SCORED BEHAVIOR. (CD_{50}= 41 mg/kg; CL = 33 - 50)

(*5 MICE TREATED WITH 70 mg/kg BNMPA AND 9 MICE TREATED WITH 80 mg/kg BNMPA DIED AFTER EXHIBITING SEIZURES)

SCORE	SALINE CONTROLS	BNMPA (mg/kg i.p.)				
		10	30	50	70	80
0	8	8	8	3	1	0
1	2	2	0	0	0	0
2	0	0	1	0	0	0
3	0	0	0	0	0	0
4	0	0	1	7	9*	10*

TABLE 10. CONVULSANT BEHAVIOR IN MICE FOLLOWING INTRAPERITONEAL INJECTIONS OF METH. NUMBERS ARE NUMBER OF ANIMALS OUT OF A GROUP OF 10 THAT EXHIBITED EACH SCORED BEHAVIOR.
(* THESE MICE DIED SUBSEQUENT TO EXHIBITING CONVULSIONS).

SCORE	SALINE CONTROLS	S(+)-METH (mg/kg i.p.)			
		10	30	50	70
0	8	5	6	4	2
1	2	2	1	1	0
2	0	3	3	4	2
3	0	0	0	0	0
4	0	0	0	0	6*

TABLE 11. CONVULSANT BEHAVIOR IN MICE OBSERVED FOLLOWING INTRAPERITONEAL INJECTIONS OF COMBINATIONS OF METH AND BNMPA .
THE FIRST COLUMN UNDER EACH DOSE IS THE DOSE OF METH ALONE;
THE SECOND COLUMN UNDER EACH DOSE IS THAT DOSE OF METH COMBINED WITH 10 mg/ kg BNMPA.
NUMBERS ARE NUMBER OF ANIMALS OUT OF A GROUP OF 10 THAT EXHIBITED EACH SCORED BEHAVIOR.

SCORE	SALINE CONTROLS		1 mg/kg S(+)-Meth		3 mg/kg S(+)-Meth		5 mg/kg S(+)-Meth		10 mg/kg S(+)-Meth	
	Alone	BNMPA	Alone	BNMPA	Alone	BNMPA	Alone	BNMPA	Alone	BNMPA
0	8	9	1	6	3	6	4	5	4	5
1	2	1	3	3	0	0	0	0	0	0
2	0	0	5	1	7	4	6	5	6	5
3	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0

In the interaction study, there was also a significant effect of convulsions [$X^2(9)=23.3$, $p< 0.05$]. However, as can be seen in Table 10, BNMPA had little influence on METH-induced convulsions. One animal in the 1 mg/kg METH alone group died after exhibiting convulsions.

7.5 Conclusions

Structurally, BNMPA is more similar to benzphetamine than either methamphetamine or amphetamine. Benzphetamine is a sympathomimetic amine that represented an attempt to produce a drug with anorectic properties while decreasing the central stimulant properties of this class of drug (Brooks et al., 1982). Among the major behavioral effects of stimulants such as amphetamine, the only one found to be mediated by a non-dopaminergic projection was anorexia (Robbins and Sahakian, 1983). Because of this structure-activity relationship, it is not surprising that BNMPA produced convulsant behavior and lethality but failed to elicit locomotor stimulation. Although it is possible BNMPA may have had locomotor effects which were short-lived, no significant differences were observed in the first 10-minute time bin of either spontaneous activity experiment. Thus it can be concluded that not only does BNMPA's structure predispose it to have a low affinity for the dopaminergic receptors responsible for locomotor activity, it may also have a greater affinity for GABA, glutamate (NMDA) or serotonergic ("seizurgenic") receptors. In one reported case of benzphetamine poisoning (Brooks et al., 1982) autopsy findings suggested a significant convulsant episode prior to death with the cause of death being circulatory collapse.

Based on the acute toxicity studies, it was expected that spontaneous convulsions would have been induced in at least 50% of the animals when dosed at 50 mg/kg in the spontaneous activity experiments. These may not have been noted in this study since the animals cannot be observed while they are in the activity chambers.

While BNMPA appears to have toxic effects in the CNS, the failure to affect locomotor activity or alter METH-induced increases in spontaneous activity or convulsions suggests that the two agents are producing their effects through distinct mechanisms.

Chapter 8

In vitro Pharmacological Characterization of α-Benzyl-N-Methylphenethylamine (BNMPA), at Selected Central Nervous System Binding Sites

8.1 Abstract

BNMPA (α-benzyl-N-methylphenethylamine), an impurity of illicit methamphetamine synthesis, has previously been reported to produce convulsions in mice (CD_{50} = 41 mg/kg) without affecting spontaneous locomotor activity or altering either methamphetamine (METH)-induced increases in spontaneous activity or METH-induced convulsions. In this study the *in vitro* effects of BNMPA on a variety of neuronal receptor types was determined in order to better characterize the pharmacological actions of this compound. BNMPA and N-demethyl-BNMPA displaced the dopamine transporter selective ligand ^{3}H-CFT (2-β-carbomethoxy-3-β-(4-fluorophenyl)tropane-1,5-napthalene disulfonate; "WIN-35428") from rat striatal membranes with K_i values (mean ± S.E.M.) of 6.05 μM ± 0.15 and 8.73 μM ± 1.66, respectively. The basal efflux of tritiated dopamine from striatal slices was enhanced by BNMPA only at concentrations of 100 μM and greater. BNMPA had no effect on tritiated norepinephrine efflux from hippocampal slices. BNMPA fully inhibited the binding of the serotonin transporter selective ligand ^{3}H-paroxetine (PXT) to cortical membranes with a K_i of 14.5 μM ± 6.8. N-Demethyl-BNMPA, (≤100 μM) failed to

produce at least 50% displacement of PXT binding. BNMPA fully displaced the α_1-receptor (α_1AR) selective ligand ^{3}H-prazosin (PRZ) from whole brain membranes with a K_i (± SEM) of 11.7 µM. Finally, BNMPA did not alter GABA induced currents in cultured cortical neurons but significantly inhibited NMDA-activated currents in oocytes expressing the NR1/2A or NR1/2C receptor subunit combinations with IC_{50}'s of 24.6 ±1.8 and 24.0 ±1.5 µM respectively. The BNMPA-induced blockade of NMDA currents was voltage-dependent with increased blockade at more negative potentials. These results suggest that BNMPA may have multiple sites of action in the CNS that could be important in modulating the behavioral effects of methamphetamine contaminated with this byproduct.

8.2 Introduction

The behavioral effects of stimulants like amphetamine and cocaine on locomotor activity appear to result from their interaction with dopaminergic systems (Kuczenski, 1983; Robbins and Sahakian, 1983; Johanson and Fischman, 1989). Seizures induced by stimulants are generally associated with non-dopaminergic systems (Ritz and George, 1993; Bloom, 1985). In a previous study (Chapter 7), BNMPA failed to significantly affect locomotor activity or alter METH-induced increases in spontaneous activity suggesting that BNMPA does not behave as a direct or indirect dopaminergic agonist. However, BNMPA produced spontaneous tonic-clonic convulsions at doses lower than those causing lethality (CD_{50}; 41 mg/kg; LD_{50}; 70 mg/kg). Noggle et al. (1985) also reported similar values for the seizure producing effects for BNMPA (CD_{50}; 54 mg/kg) and N-demethyl-BNMPA (CD_{50}; 45 mg/kg). In METH-interaction studies,

while we did note that BNMPA appeared to have a small paradoxical neuroprotective effect against low-dose METH-induced convulsions, there was no significant alteration of METH-induced convulsions.

Based on these *in vivo* observations and BNMPA's structural similarity to benzphetamine (an anorexiant designed to minimize other CNS effects), we hypothesized that BNMPA should have a relatively low affinity for dopaminergic systems involved in locomotor activity, while its seizure-producing effects may involve an interaction with GABAa receptors, glutamate / N-methyl-D-aspartate (NMDA) or serotonergic receptors.

In this study, the effects of BNMPA on these receptor systems was investigated to better understand the molecular and cellular effects of this compound.

8.3 Materials and methods

8.3.1 Animals

Male Sprague-Dawley rats (250-300 g) were obtained from Harlan (Dublin, Virginia). Water and food (Rodent Laboratory Chow, Ralston-Purina Co., St. Louis, MO) were available *ad libitum* . Animals were housed singly in standard cages (18 x 29 x 13 cm) with wood chip bedding in a controlled temperature room (22-24° C) with a12-hr light-dark cycle. All experimental protocols were approved for use by the university IACUC committee and conform to NIH guidelines for use of laboratory animals.

8.3.2 Chemicals and reagents

^{3}H-CFT (83.4 Ci/mmol), ^{3}H-paroxetine (^{3}H-PXT; 16.6 Ci/mmol) and ^{3}H-prazosin (^{3}H-PRZ; 76 Ci/mmole) were obtained from New England Nuclear

(Boston, MA). Cocaine hydrochloride (NIDA) and paroxetine hydrochloride hemihydrate (SmithKline Beecham Pharmaceutical) were generous gifts. Phentolamine was obtained from Sigma Chemicals, St. Louis, Missouri (Catalogue #P7547). BNMPA and N-demethyl-BNMPA were synthesized as described in Chapter 3. All other chemicals and reagents were obtained from Sigma Chemicals.

8.3.3 Data analysis

K_D, K_i and B_{max} were determined using the KELL software package EBDA program (Biosoft, Milltown, NJ). Data are expressed as ± SEM. Figures are of a single representative experiment done in triplicate, therefore error bars are not shown. Reported means are from n=3 - 5. Dose response curves were analyzed using the ALLFIT program with the minimum value set at zero.

8.3.4 Dopamine/serotonin transporter binding assays

^{3}H-CFT binding. CFT binding was conducted using the method described by Woodward et al. (1995), using striatal tissue.

^{3}H-Paroxetine binding. Paroxetine binding was conducted using a modified Habert et al. (1985) and Mann and Hrdina (1992) protocol. Briefly, animals were decapitated and cortical tissue removed and placed in 20 ml ice-cold buffer (50 mM Tris-HCl, 15 mM NaCl, 5 mM KCl, pH 7.4). Tissues were homogenized using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) and centrifuged at 40,900 xg at 4°C for 20 minutes. Supernatant was decanted and 20 ml of ice-cold buffer added, tissue re-homogenized and centrifuged as before. Supernatant was again decanted, the tissue diluted to its reaction volume (40 mg tissue/ml buffer) and aliquotted into the reaction tubes immediately. After one hour incubation at 22°C, tissue binding was terminated by

the addition of 2 ml of ice-cold buffer and filtered onto a 0.05% PEI pretreated Whatman GF/B filter using a Brandel 48 well harvester (Gaithersburg, MD). Filters were placed in scintillation vials containing 10 ml Bio-Safe II® scinitillation cocktail (RBI) and radioactivity quantitated on a Beckman LS 6000 scinitillation counter. Protein content of tissues was determined spectrophotometrically using the Biorad dye technique for a modified Lowry's protein assay.

Neurotransmitter release assay. The release of preloaded tritiated dopamine and norepinephrine (NA) from striatal and hippocampal slices was determined as described previously (Woodward and Blair, 1991). Briefly, slices were incubated in 37°C bubbled (95% O_2 / 5% CO_2) Krebs-Ringer bicarbonate buffer (in mM): NaCl (118), KCl (4.7), $NaHCO_3$ (24.8), KH_2PO_4 (1.18), $CaCl_2$ (1.0), glucose (10), HEPES (25), pH 7.4. After one hour of washing, slices were incubated for 30 minutes in the presence of 100 nM ^{3}H-NA or ^{3}H-DA (Amersham, Arlington Heights IL). Pargyline (a monoamine oxidase inhibitor, 20 µM) and ascorbic acid (an antioxidant, 1 mM) were present during the loading period to protect the labeled catecholamine from degradation and metabolism. Following loading, slices were washed, loaded into individual nylon mesh-bottomed baskets (210 µm nylon mesh, Tetko Inc., Elmsford NY) and suspended in mini-vials each containing 3 mLs of buffer. Baskets were transferred at two-minute intervals with continuous bubbling with 95% O_2 / 5% CO_2 through a series of vials. BNMPA (10-300 µM) was present in the last row of vials. Following the drug exposure, the tissue was lysed by incubating in 0.1 M perchloric acid for 1 hour to release all of the neurotransmitter remaining in the slices. The radioactivity contained in the vial was quantitated by liquid

scintillation spectrometry. The fractional release of neurotransmitter in each vial was calculated as a percent of the total neurotransmitter present at that time.

8.3.5 α_1-Receptor binding assay

Tissue homogenate preparation. Brain homogenates for saturation and competitive binding experiments were made by introducing one whole male, Sprague-Dawley rat brain into a large tissue homogenizing tube, adding 50 ml. ice cold cell buffer (50 mM Tris, 10 mM $MgCl_2 \cdot 6H_2O$; pH 7.4) and homogenizing with a Teflon pestle for 1 minute or until a dispersed homogenate was obtained. Initial determination of total protein was done by assay with BioRad Protein Dye. Protein was adjusted to 150-200 µg/ml in cell buffer by dilution of the primary homogenate. Homogenates were stored on ice until use.

Competitive binding. Stock solutions of 1×10^{-3} M, 3×10^{-4} M, 1×10^{-4} M, 3×10^{-5} M and 1×10^{-5} M BNMPA in the presence and absence of 10^{-4} M phentolamine were prepared in cell buffer. ^{3}H-PRZ stock (10 nM) was made to provide a single saturating concentration of 1 nM in the final reaction mixture. One-hundred microliters of stock solutions of BNMPA in the presence or absence of phentolamine as described above, 100 µl of ^{3}H-PRZ stock and 800 µl of rat brain homogenate were added to glass culture tubes. Tubes were incubated at 31° C in a shaking water bath for 50 minutes and filtered onto a 24 well Brandon cell harvester. Filters were introduced into 7 ml scintillation vials, to which was added 5 ml of Scintisafe-Plus® 50% (Fisher SX25-5) and counted on a Beckman LS6000 Beta Counter.

Saturation binding. A stock solutions of 10^{-4} M ^{3}H-PRZ was diluted to concentrations ranging from 0.625 to 10 nM in cell buffer. One-hundred microliters of cell buffer alone or with 10^{-4} M phentolamine stock was combined

with 100 µL of ^{3}H-PRZ at the various dilutions described above and 800 µL rat brain homogenate in culture tubes. Tubes were made in quadruplicate and assayed according to the method described above.

8.3.6 NMDA receptor studies

The NMDA receptor clones were generous gifts of Dr. S. Nakanishi (Kyoto, Japan). mRNA synthesis, oocyte preparation and micro injection were done as described previously (Mirshahi and Woodward, 1995).

Electrophysiological recordings and data analysis were carried out as described with minor modifications (Mirshahi and Woodward, 1995). All recordings were done in Mg^{2+}-free Ringers with Ba^{2+} as the divalent charge carrier. For the dose response determinations, oocytes were stimulated by switching to a perfusion solution containing NMDA and glycine and various concentrations of BNMPA for 20 sec using a six-port injection valve. Each stimulation was preceded by a washout period of 3-5 min. For the voltage dependence studies, oocytes were ramped from -80 to +40 mV in 2 sec and leak currents were subtracted under each condition. All stimulations were done using 100µM NMDA and 10 µM glycine unless otherwise stated. Experiments were done at room temperature (20-22°C).

8.4 Results

8.4.1 ^{3}H-CFT binding/neurotransmitter release

Radiolabeled CFT membrane binding to the naive rat striatum yielded a linear Scatchard plot with a mean K_D value of 37.95 ± 7.16 nM and B_{max} 3.69 ± 0.96 nM/mg. In the striatum, ^{3}H-CFT binds to a single population of receptor sites. Displacement studies with BNMPA and N-demethyl-BNMPA yielded

K_i values (mean ± SEM) of 6.05 µM ± 0.15 and 8.73 µM ± 1.66, respectively (Figure 18). The basal efflux of tritiated dopamine from rat striatal slices averaged approximately 2.8% (± 0.2) per two minute period. BNMPA at concentrations of 10 and 30 µM did not significantly alter basal dopamine efflux. Higher concentrations of BNMPA (100 and 300 µM) increased the basal efflux of tritiated dopamine to 4.5% and 8.7% respectively. The basal efflux of tritiated norepinephrine from rat hippocampal slices averaged 0.8% (± 0.04) per two minute period. BNMPA did not significantly alter the efflux of tritiated norepinephrine at any concentration tested (10-300 µM).

8.4.2 ^{3}H-Paroxetine binding

Paroxetine membrane fragment binding to cortical tissue of naive rat yielded a linear Scatchard plot with a mean K_D value of 548 ± 81.07 pM and B_{max} 121.33 ± 4.04 pM/mg. The ^{3}H-paroxetine binding to the neuronal membranes from the cortex seemed to indicate a single population of receptor sites. BNMPA had an estimated K_i of 14.5 µM ± 6.83 (Figure 19). The K_i value for N-demethyl-BNMPA could not be obtained since the maximum displacement obtained was 49% (mean= 42% ± 2.6) at 100 µM of drug .

8.4.3 ^{3}H-Prazosin binding

Prazosin membrane fragment binding to whole brain of naive rat yielded a linear Scatchard plot with a mean K_D value of 251 pM and B_{max} 10 pM. The ^{3}H-PRZ binding to the neuronal membranes seemed to indicate a single population of receptor sites. Displacement studies with BNMPA yielded a K_i value of 11.7 µM (Figure 20).

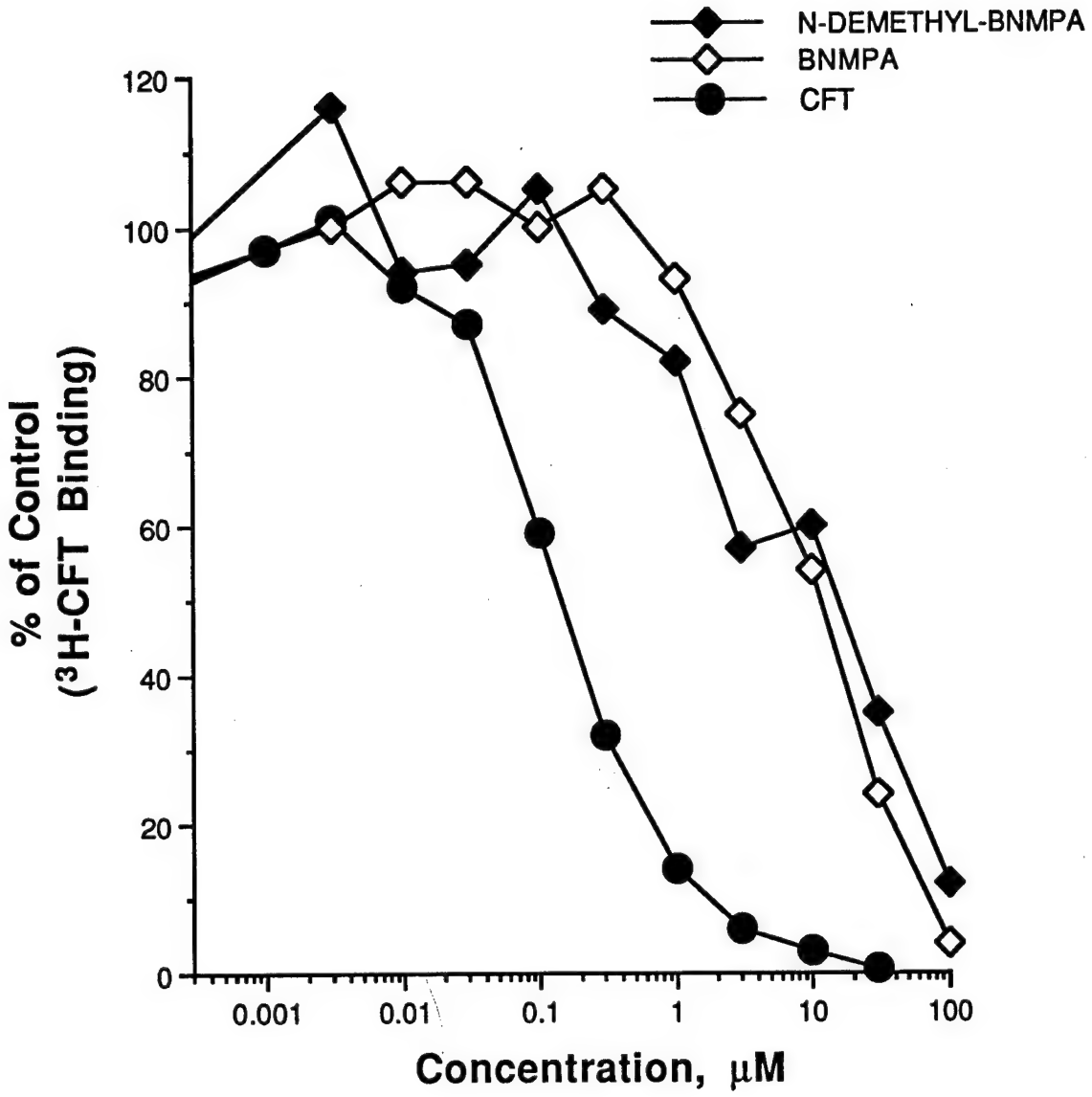


FIGURE 18. DISPLACEMENT OF ^{3}H-CFT BINDING (30 nM) IN FRESH RAT STRIATUM BY N-DEMETHYL-BNMPA, BNMPA AND CFT. EACH CURVE IS ONE REPRESENTATIVE OF EXPERIMENTS DONE IN TRIPLICATE.

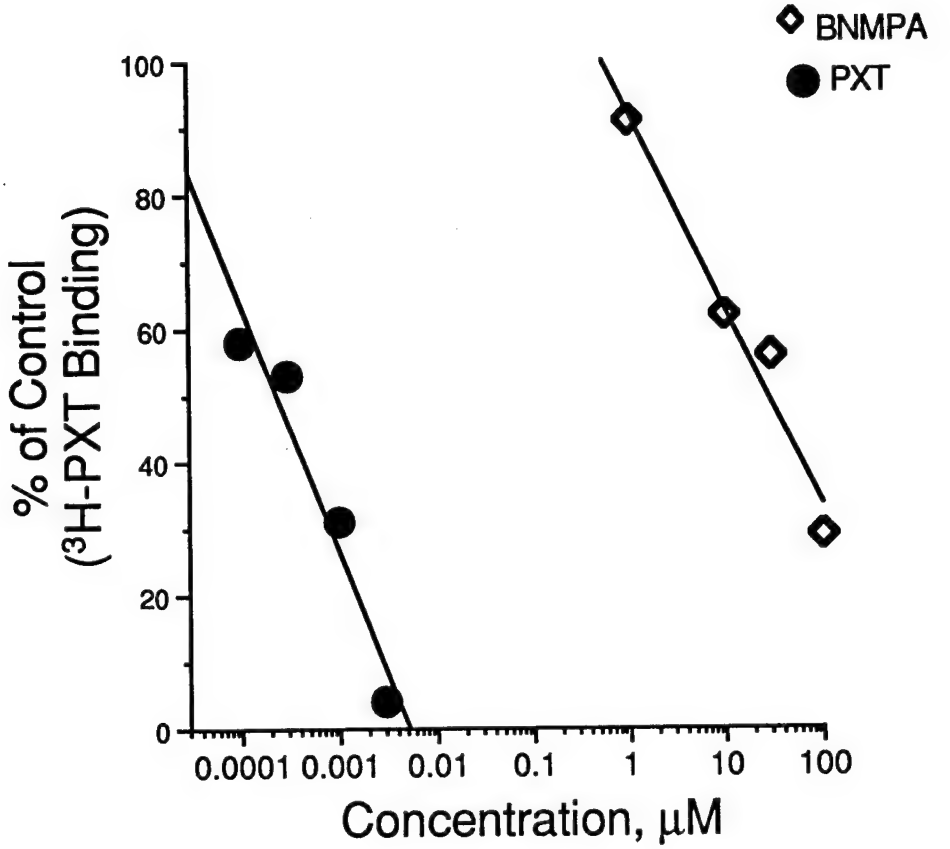


FIGURE 19. DISPLACEMENT OF ^{3}H-PXT BINDING (1.5 nM) IN FRESH RAT CORTEX BY BNMPA AND PXT. THE K_i VALUE FOR N-DEMETHYL BNMPA COULD NOT BE OBTAINED SINCE THE MAXIMUM DISPLACEMENT OBTAINED WAS 49% (MEAN = 42% ± 2.6) AT 100 μM OF DRUG. EACH CURVE IS ONE REPRESENTATIVE OF EXPERIMENTS DONE IN TRIPLICATE.

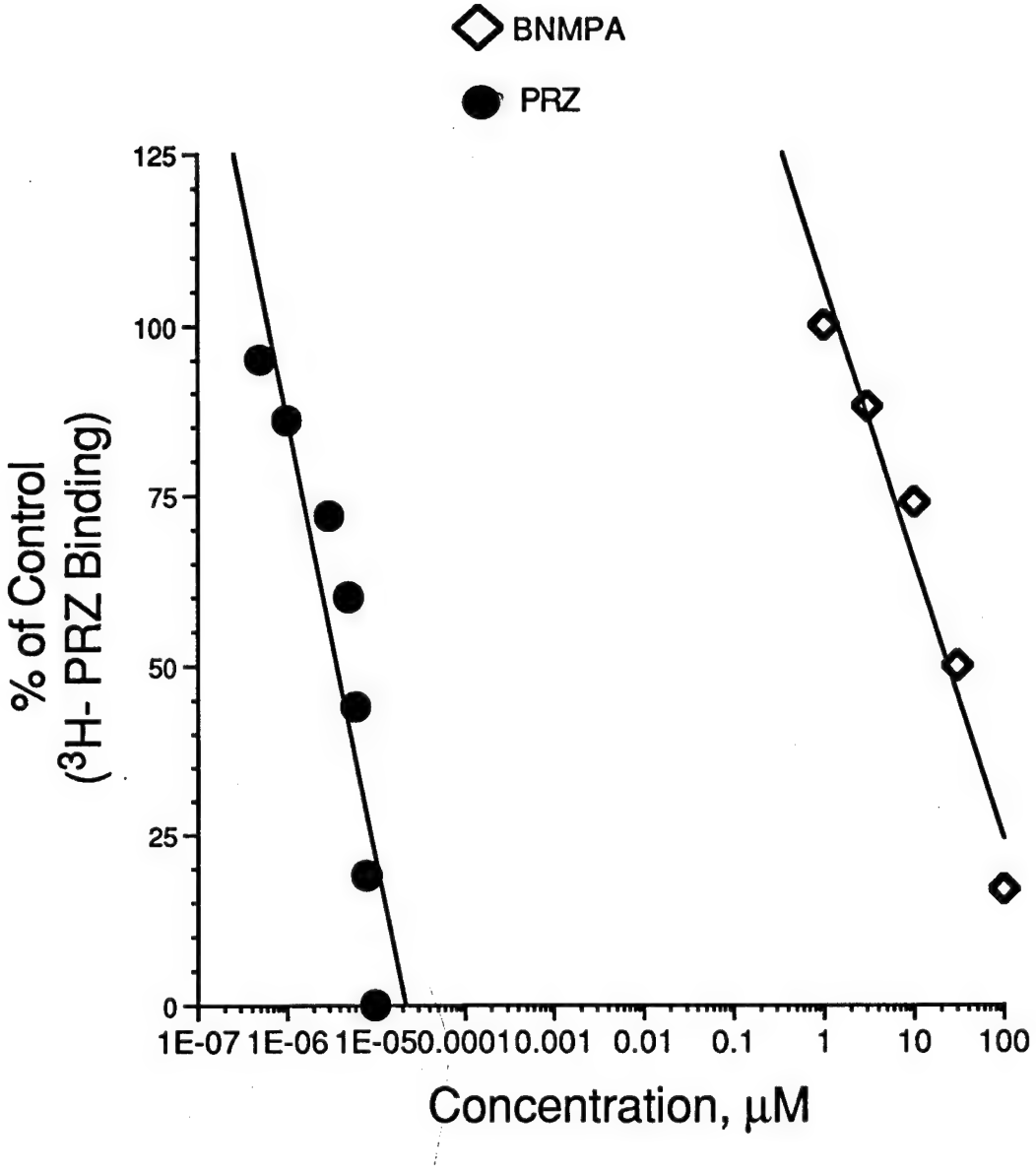


FIGURE 20. DISPLACEMENT OF ^{3}H-PRZ BINDING (1 nM) IN FRESH RAT WHOLE BRAIN BY BNMPA AND PRZ. EACH CURVE IS ONE REPRESENTATIVE OF EXPERIMENTS DONE IN QUADRUPLICATE.

8.4.4 NMDA receptor studies

Application of BNMPA alone did not induce any currents in uninjected oocytes or oocytes expressing heteromeric NMDA receptors. Figure 21 shows that 100 μM BNMPA inhibits the NMDA-induced currents in oocytes expressing the NR1/2A or NR1/2C receptors subunits. The washout currents were always smaller than the previous controls indicating that some of the drug remains on the receptor complex even after several minutes of wash out. The BNMPA inhibition of the NMDA-activated current is slowly reversible, most of the current recovers after minutes of washout. Figure 22 shows dose response curves for the BNMPA inhibition of NMDA-induced currents in oocytes expressing the NR1/2A or NR1/2C subunits. The IC_{50}'s for BNMPA in oocytes expressing the NR1/2A and NR1/2C were 24.6±1.8 and 24.0±1.5 μM respectively. Near maximal inhibition was seen for both subunit combinations at 300μM BNMPA. The inhibitory effects of BNMPA were not altered by increasing the concentration of NMDA or glycine (data not shown).

Figure 23 shows the current voltage relation for the BNMPA block of the NMDA-activated currents in oocytes expressing the NR1/2A or NR1/2C subunits. The BNMPA block of the NMDA-activated current in both subunit combinations displayed a strong voltage dependence. The NMDA-activated current was inhibited by BNMPA at all holding potentials but a higher level of inhibition was seen in the lower holding potentials. BNMPA also altered the reversal potential of the NMDA-activated currents in both receptor subunit combinations. The reversal potentials were approximately -18 mV for both NR1/2A and NR1/2C and approximately -5 mV in the presence of BNMPA .

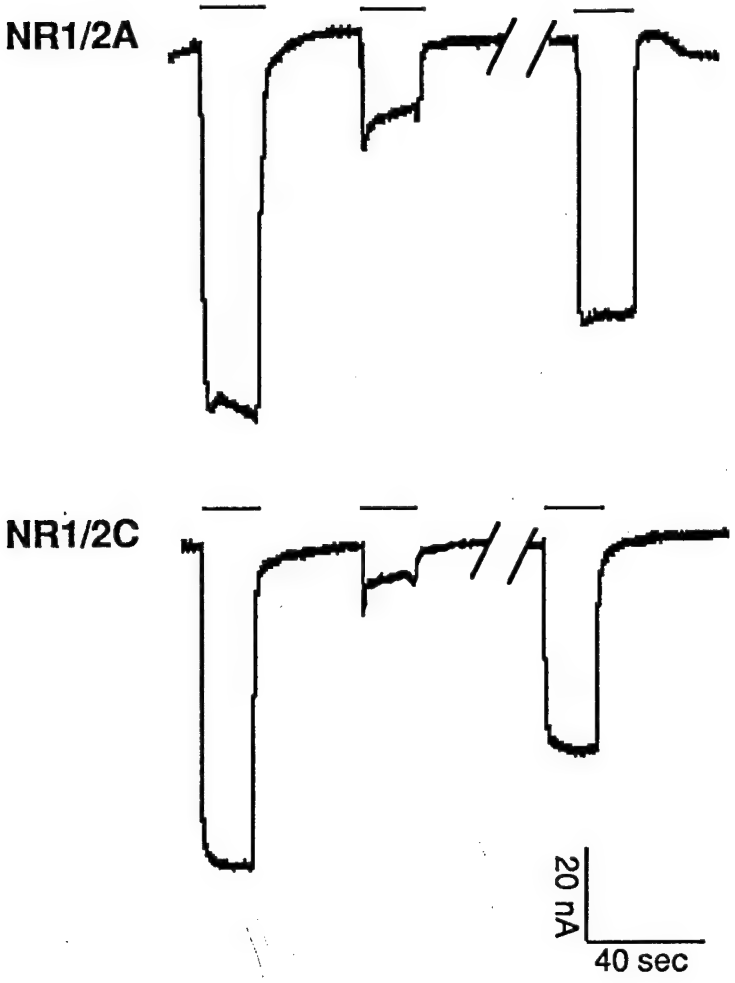


FIGURE 21. EFFECTS OF 100 μM BNMPA ON NMDA-ACTIVATED CURRENTS. OOCYTES EXPRESSING NR1/2A (TOP) AND NR1/2C (BOTTOM) NMDA RECEPTOR SUBTYPES STIMULATED WITH 100 μM NMDA AND 10 μM GLYCINE FOR 20 SECONDS IN THE PRESENCE AND ABSENCE OF 100 μM BNMPA. BNMPA INDUCES A RAPID INHIBITION OF THE NMDA-ACTIVATED CURRENTS WHICH IS SLOWLY REVERSIBLE.

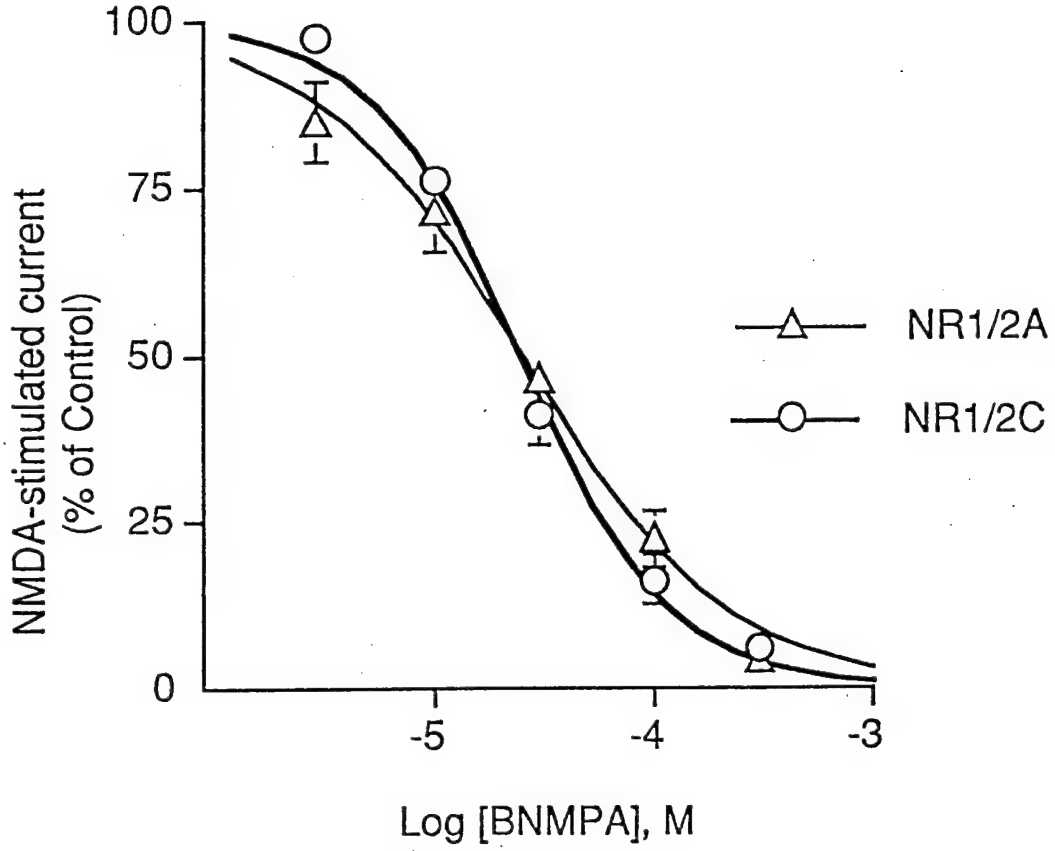
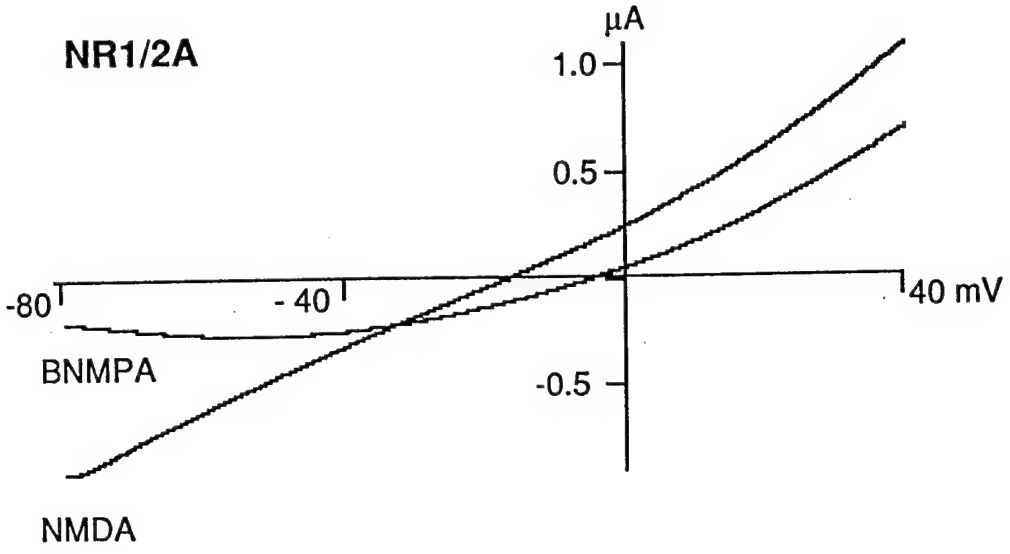


FIGURE 22. DOSE-RESPONSE CURVES OF BNMPA-INDUCED INHIBITION OF NMDA-ACTIVATED CURRENTS IN OOCYTES EXPRESSING THE NR1/2A AND NR1/2C NMDA RECEPTOR SUBUNITS. ALL VALUES WERE OBTAINED IN TH PRESENCE OF 100 μM NMDA AND 10 μM GLYCINE. BNMPA INHIBITS THE NR1/2A AND NR1/2C RECEPTOR COMBINATIONS WITH IC_{50}'s OF 24.6 ± 1.8 AND 24.0 ± 1.5 μM, RESPECTIVELY.



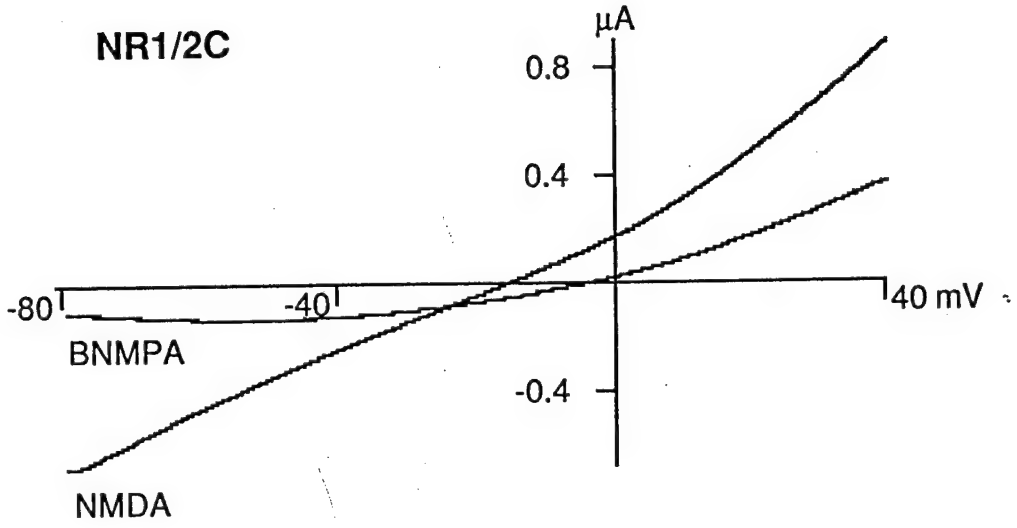


FIGURE 23. CURRENT VOLTAGE DEPENDENCE OF THE BNMPA BLOCK. OOCYTES EXPRESSING THE NR1/2A AND NR1/2C RECEPTORS WERE ACTIVATED BY APPLICATION OF 100 µM NMDA AND 10 µM GLYCINE IN THE ABSENCE AND PRESENCE OF 100 µM BNMPA. CURRENT-VOLTAGE CURVES WERE GENERATED BY 2 SECOND RAMPS FROM -80 mV TO +40 mV; LEAK CURRENTS WERE SUBTRACTED IN EACH CASE.
BNMPA DISPLAYS A VOLTAGE DEPENDENT BLOCK OF THE NMDA-ACTIVATED CURRENTS WITH MORE BLOCKADE AT LOWER POTENTIALS. SHIFTING OF THE REVERSAL POTENTIALS OF THE CURRENTS ALSO OCCURS WHICH IS INDICATIVE OF A CHANGE IN THE ION SELECTIVITY OF THE CHANNELS.

8.5 Conclusions

Doses of BNMPA ranging from 1 mg/kg to 50 mg/kg failed to significantly alter locomotor activity significantly from controls (Chapter 5). Measurement of spontaneous activity when BNMPA was combined with METH was not significantly different from METH alone. Based on these observations, we predicted that BNMPA / metabolites should have minimal interaction at dopaminergic sites. This was partially confirmed in this study. While BNMPA and N-demethyl-BNMPA's affinity at the dopamine transporter was low relative to CFT (mean K_i of 6.05 μM for BNMPA vs. a K_D of 37.95 nM for CFT), the K_i for (-)-cocaine at the dopamine transporter is only tenfold greater at 0.54 μM (Ritz and George, 1993). Assuming complete distribution in body water, we estimated the *in vivo* convulsive "concentration" of BNMPA in a 20 g. mouse to be 368 μM (50 mg/kg) (Chapter 7). Because BNMPA completely displaced CFT at much lower concentrations (100 μM) and is so close to the K_i for cocaine, it is interesting to speculate that compounds such as BNMPA may have pharmacological application as a compound that can displace cocaine from the dopamine transporter without producing undesirable side effects such as locomotor stimulation, convulsions and the rewarding effects that lead to substance abuse.

The results of the studies on the effect of BNMPA on the NMDA-activated currents in the oocytes indicate potent blocking activity of BNMPA on this receptor channel. BNMPA blocked NMDA-activated currents in both NR1/2A and NR1/2C subunit combinations of the NMDA receptor expressed in oocytes. BNMPA does not seem to compete with either NMDA or glycine for a binding site on the receptor since the potency of BNMPA was unchanged in the presence of

different concentration of NMDA and/or glycine. BNMPA blockade of the NMDA-activated current displays a strong voltage dependence. This blockade is very similar to the well characterized Mg^{++} blockade of the NMDA-activated current. However, when compared to the Mg^{++} blockade, the BNMPA block is slightly less voltage dependent with residual block even at positive holding potentials. It has been shown that NMDA receptor blockers posses neuroprotective properties and may serve as effective anticonvulsant agents. BNMPA can block the NMDA receptor at pharmacologically relevant concentrations and should serve as an anticonvulsant in the micromolar concentration range. Sonsalla et al. (1989) reported that the noncompetitive NMDA receptor antagonist, MK-801, as well as other glutamate receptor antagonists protect the mouse neostriatal dopaminergic system from neuronal degeneration induced by METH. This is consistent with our data indicating that BNMPA suppresses low-dose METH-induced convulsions.

BNMPA has been shown to be seizurgenic (Noggle et al., 1985; Chapter 5). Drugs can increase excitability in the CNS (be "seizurgenic") either by blocking inhibition or enhancing excitation (Franz, 1985). The primary inhibitory neurotransmitter in the CNS is gamma-aminobutyrate (GABA). Many convulsants (e.g., pentylenetetrazol and picrotoxin), have been found to be selective antagonists of GABA (Bloom, 1985). Glutamate and aspartate are found in very high concentrations in the brain and both are very powerful excitatory neurotransmitters. Glutamate activation of the N-methyl-D-aspartate (NMDA) receptors leads to a rapid and significant increase in intracellular calcium. Elevation in intracellular calcium is believed to be the primary event in neuronal death observed in cell culture models of glutamate-mediated excitotoxicity (Choi,

1992). Additionally, in studies with cocaine, Ritz and George (1993) demonstrated that the potencies of cocaine and related drugs in producing seizures appear to be highly associated with drug affinity at serotonin transporters. However, BNMPA and N-demethyl-BNMPA displayed low affinity for the serotonin transporter (K_i=14.5 μM for BNMPA vs. a K_D=548 pM for PXT). Additionally, BNMPA does not appear to have any effects on GABA-activated currents in dissociated rat cortical neurons[1].

The studies conducted for this preliminary report on the molecular basis of the actions of BNMPA failed to account for its convulsive activity. Convulsions are a common sequelae following long-term/high-dose use of stimulants such as cocaine, the amphetamines, amphetamine analogs (MDMA, MDA, etc.), pemoline, aminorex and some new stimulants of abuse such as 4-methylaminorex ("u4euh") (Franz,1985; Bunker, et al., 1990; Ritz and George, 1993). The mechanisms of seizure induction appear to be as varied as the drugs themselves, however, serotonin depletion has been implicated in several models of convulsions, neurotoxicity and clinical syndromes such as depression, multiple sclerosis, schizophrenia and "stimulant psychosis" (Rudnick and Clark, 1993). Depletion of the amine in brain increases, whereas agents that increase 5-HT concentration decrease susceptibility to seizures (Przegalinski, 1994). Stimulants achieve this "final common pathway" to seizurgenesis/serotonin reduction through a variety of mechanisms including decreased synthesis, decreased release, increased metabolism or death of serotonergic neurons.
Even though BNMPA and its N-demethyl metabolite showed little affinity for the

[1]Personal communication, Douglas Coulter, Ph.D., Department of Neurology, Medical College of Virginia.

serotonin transporter, they, like their structurally analogous anorexiants, may inhibit TPH activity, cause direct cell death or be a 5-HT_{1C} or 5-HT_2 receptor antagonist. Additionally, since BNMPA showed some affinity for the α_1-receptor,. it may be acting as an antagonist at this site, thus contributing to its convulsant nature.

Chapter 9

General Discussion

The purpose of this dissertation was fivefold: first, to predict the metabolites of α-benzyl-N-methylphenethylamine (BNMPA), an impurity of illicit methamphetamine synthesis, and synthesize them as well as BNMPA; secondly, to develop a detection method for these compounds in biological fluids; third, to confirm the predicted metabolites in a metabolic study with humans; fourth, to confirm the utility of these compounds as indicators of illicit methamphetamine consumption by establishing their presence in biological samples from known methamphetamine users; finally, to explore the *in vivo* pharmacology/toxicology of BNMPA and establish a mechanism of action for any observations at the molecular level using various *in vitro* pharmacological assays.

Increasing abuse of stimulants such as methamphetamine in the decades following World War II, led to their classification as a Schedule II controlled substance under the Controlled Substances Act (Public Law 91-513) of 1970, limiting methamphetamine's acquisition through legitimate channels. This Act still recognized that many controlled drugs have a useful medical purpose and established a system of annual registration, under which a firm is registered to manufacture or import a limited amount of controlled drugs. It is expected that legally manufactured controlled drugs are "pure" compounds and have undergone the same rigorous testing and characterization of pharmacological

and toxicological effects as any pharmaceutical approved by the Food and Drug Administration.

However, because of the manufacturing restrictions imposed by the Act, an active underground pharmaceutical industry sprang up to meet the increasing demand of abusers. Methamphetamine is currently the most frequently encountered clandestinely produced controlled substance in the United States; the Drug Enforcement Administration participated in 310 methamphetamine laboratory seizures in 1991, mainly on the West Coast and in Houston (Bono, 1993). Not coincidentally, methamphetamine is also among the twenty most frequently mentioned drugs in emergency room patients (DAWN, 1991). Illicitly manufactured methamphetamine usually contains various contaminants which may be contributing to this toxicity. In contrast to legitimate pharmaceutical manufacturers, the quality of "manufacturing personnel" in the underground industry varies from those with no educational background merely "cookbooking" a recipe to very skilled and probably highly educated chemists. Consequently, methamphetamine produced in clandestine laboratories often contains impurities arising from incomplete reaction and inadequate purification of intermediates and/or the final product of the synthesis. The substance that finally makes it to the streets, is not only impure, but often contains other substances purposefully added as diluents and adulterants (Soine, 1989). The presence of diluents and adulterants is highly variable, however the impurities of manufacture are usually present and vary only in concentration.

In 1993, the DEA participated in the seizure of 270 clandestine laboratories in the United States. Of these laboratories, 218 were methamphetamine labs. The ephedrine reduction method was used in 81% of all

methamphetamine labs seized during 1993, whereas the P2P method was used for only 16% (DEA, 1994). This represents a direct reversal of trends noted in the 1980's (Soine, 1986). Forensic laboratories in New Mexico also reported that in the first six months of 1994, there was a "decided increase" in the number of cases of methamphetamine seizures, all ephedrine process (*Microgram,* 1994). Additionally, the California Bureau of Narcotic Enforcement reports that the use of the precursor chemical ephedrine in California has rapidly increased with the availability of the chemical from Mexico. Word of the value of ephedrine as a precursor in methamphetamine manufacture has spread quickly in the Mexican drug community , causing it to become a "significant smuggling commodity" and the price to drop in the U.S. (Largent, 1994).

This 180º reversal in illicit synthetic methods may account for the fact that 2 out of the 40 "older" samples that were analyzed from methamphetamine abusers contained BNMPA and metabolites while none of the samples collected and analyzed since mid-1994 contained this impurity.

In March, 1994, the Federal Register documented the proposal by the DEA to make all regulated transactions of ephedrine, regardless of size, subject to the reporting and record keeping requirements of the Chemical Diversion and Trafficking Act of 1988 (CDTA). This would subject all transactions involving bulk ephedrine and single entity ephedrine drug products to the applicable provisions of the CDTA (*Federal Register,* 1994). The CDTA has reportedly already made the acquisition of commercially produced HI much more difficult. The illicit price of a gallon of HI in California has increased sharply to between $4000 and $7000. Lack of availability and this price increase has made violators seek alternative sources of HI and/or different processes of clandestinely

manufacturing methamphetamine (*Microgram*, 1993). We believe the addition of tightening of ephedrine controls will soon shift the pendulum back to some of the "older" methods of methamphetamine synthesis and the impurities of synthesis such as BNMPA will once again become legally significant. A recent issue of "Microgram" (1995) contained the following:

> "The Sacramento County, California District Attorney Laboratory of Forensic Services reported receiving a submission of a powder suspected of being methamphetamine...The Marquis test gave a 'flash of purple followed by the usual orange color' and the nitroprusside test was consistent with a secondary amine (blue color). Analysis by FTIR and GC/MS, however, did not reveal the presence of any methamphetamine. Rather, the powder turned out to be essentially pure α-benzyl-N-methylphenethylamine".

It was confirmed that BNMPA / metabolites are present in sufficient quantities to serve as a marker of illicit consumption in the previous 24 hours of methamphetamine synthesized via reductive amination procedures using P2P synthesized from phenyl acetic acid.

Investigations into the potential toxicity of BNMPA were less conclusive. One of the defining characteristics of psychomotor stimulants (METH, AMPH, cocaine, etc.) is their ability to elicit increases in spontaneous motor activity. This was observed in our study with METH and AMPH but not BNMPA. Although it is possible BNMPA may have had locomotor effects which were short lived, no significant differences were observed in the first 10-minute time bin of either spontaneous activity experiment.

This study may not be representative of what can be expected in true METH abusers. The primary routes of administration for METH in humans are intravenous and smoking. Both of these routes allow a substantial amount of drug to bypass "first-pass" metabolism whereas intraperitoneal injections do not.

Intraperitoneal injections were chosen for this study since this route of administration has the largest data base with which to compare this relatively unstudied compound. Even with intraperitoneal injection and "first-pass" metabolism, methamphetamine would still produce its effects on spontaneous activity since the primary metabolite of methamphetamine is amphetamine. One of the important metabolic pathways of BNMPA metabolism appears to be hydroxylation/glucuronide conjugation (Chapter 4). These mechanisms tend to inactivate compounds.

Second, this study was done following a single dose of METH and BNMPA. The pattern of methamphetamine use in abusers is high doses (sometimes several thousand milligrams per day) over very long periods of time. Under these conditions, BNMPA and related lipophilic compounds (which have a much larger partition coefficient than amphetamine/methamphetamine) (Brooks et al., 1982) will be stored in tissues (including the CNS) for prolonged periods. It may be that only with extended exposure will BNMPA accumulate to pharmacologically/toxicologically significant doses.

Observations in this study suggest direction for further investigations into the pharmacology/toxicology of BNMPA. Chronic dosing studies as well as looking at other routes of administration would further characterize the role of long-term abuse in the toxicity of illicitly manufactured methamphetamine.

BNMPA produced spontaneous tonic-clonic convulsions at doses lower than those causing lethality [CD_{50} = 41 mg/kg (303 μM); LD_{50}=70 mg/kg (518 μM)] (Chapter 5). Noggle et al. (1985) also reported similar values for the seizure producing effects for BNMPA (CD_{50}; 54 mg/kg) and N-demethyl-BNMPA (CD_{50}; 45 mg/kg). In METH-interaction studies, while we did note that BNMPA

appeared to have a small paradoxical neuroprotective effect against low-dose METH-induced convulsions, there was no significant alteration of METH-induced convulsions.

Seizures, while also often associated with stimulant abuse, tend to occur only at very high doses (Weiner, 1985; Cameron et al., 1992; Ritz and George, 1993). Seizure induction is generally associated with non-dopaminergic systems (Ritz and George, 1993; Bloom, 1985). In addition to the more commonly recognized mechanisms of seizure induction(i.e., blocking inhibitory or enhancing excitatory pathways), serotonin depletion has been implicated in several models of convulsions, neurotoxicity and clinical syndromes such as depression, multiple sclerosis, schizophrenia and "stimulant psychosis" (Rudnick and Clark, 1993). Depletion of the amine in brain increases, whereas agents that increase 5-HT concentration decrease susceptibility to seizures (Przegalinski, 1994).

Stimulants achieve this "final common pathway" to seizurgenesis/serotonin reduction through a variety of mechanisms including decreased synthesis, decreased release, increased metabolism or death of serotonergic neurons. Chronic or high doses of amphetamine produce behavioral symptoms indistinguishable from schizophreniform psychosis (Sulzer and Rayport, 1990). Chronic or sub-acute administration of AMPH or METH produces prolonged decreases in regional tryptophan hydroxylase (TPH) activity (TPH is the rate-limiting enzyme in 5-HT synthesis) and concentrations of 5-HT (Trulson and Jacobs, 1979; Hotchkiss and Gibb, 1980; Trulson and Trulson, 1982). Brain TPH activity is also decreased by acute administration of AMPH and METH (Knapp, et al., 1974; Bakhit and Gibb, 1981).

Amphetamine and methamphetamine are substrates for the biogenic amine (5-HT, NE and DA) transporters and lead to transmitter release by a process of transport mediated exchange (Rudnick and Clark, 1993). Upon entry to the cytoplasm, the amphetamines further reduce accumulation of monoamines in the synaptic vesicles. Catecholaminergic vesicles use an interior-acidic proton gradient for transmitter uptake (Sulzer and Rayport, 1990). Amphetamines and other psychostimulants are lipophilic weak bases which equilibrate in pH gradients across intracellular compartments. When synaptic vesicles reach an internal pH of 5.6 in an external (cytoplasmic) medium of pH 7.4, weak bases such as amphetamine accumulate over 60-fold. After the vesicle's buffering capacity is exceeded, these compounds will induce alkalinization of the intravesicular space, reducing the driving force necessary for monoamine uptake. Weakly basic drugs would also compete for protons with neurotransmitter already present in the granules. The resulting uncharged neurotransmitter would then diffuse out of the granules down its concentration gradient. While this mechanism would cause a continuous release of neurotransmitter at low-doses of stimulant, accounting for the locomotor stimulant and "reinforcing" effects of these compounds, as the dose increases and the cytoplasmic concentrations of neurotransmitter continues to increase, the phenomena of intracellular oxidative stress becomes prominent, ultimately resulting in neurotoxicity and selective degeneration of neuron terminals without cell-body loss (Cubells, et al., 1994). When the monoamine neurotransmitters are redistributed from the reducing environment of the synaptic vesicle to extravesicular oxidizing environments, generation of oxygen radicals and reactive metabolites by monoamine oxidase within the neuron may trigger selective terminal loss. Such a mechanism could

account for direct loss of serotonergic neurons (and increased likelihood of convulsions) or decrease serotonin levels indirectly if dopaminergic input to a serotonergic system was lost. The NMDA receptor is a subclass of the glutamate receptor linked to a calcium channel which, upon stimulation, allows calcium influx into the neuron. Calcium overload is detrimental to neurons and is thought to contribute to ischemia-related damage. This "excitotoxicity" phenomena may also account for METH-induced neuronal death.

Repeated administration of large doses of the psychoactive analog of amphetamine, 3,4-methylenedioxymethamphetamine (MDMA), also destroys serotonergic nerve terminals (O'Hearn et al., 1988) which is reflected by a long-lasting decline in 5-HT and 5-HT metabolite concentrations. The regional activity of TPH and corresponding concentrations of 5-HT are dramatically reduced. Additionally, the number of high-affinity uptake sites for 5-HT are decreased. These serotonergic parameters were still significantly depressed 110 days after multiple doses of MDMA (Stone, et al., 1987). Unlike METH-induced neuronal death, MDMA neurotoxicity is not altered by MK-801 blockade of NMDA receptors (Johnson et al., 1989). This would indicate that increased calcium influx into the cell through NMDA channels and the resulting calcium overload "excitotoxicity" phenomena does not play a part in MDMA neurotoxicity. However, flunarizine, a calcium overload blocker at non-NMDA calcium channels (L- and T-type voltage-operated calcium channels), has been shown to prevent the decline in cortical and neostriatal TPH activity induced by MDMA (Johnson, et al., 1992).

4-Methylaminorex (4-MAX) was described in 1963 as an indirect-acting sympathomimetic drug with anorectic properties (Poos, et al., 1963). 4-MAX is

similar in structure to pemoline (a Schedule IV CNS stimulant) and aminorex (an anorectic marketed in Europe in 1965 but withdrawn in 1968) (Bunker et al., 1990). Recently, 4-MAX has come to the forefront as a new stimulant of abuse. Users have described the effects of 4-MAX to be like those of amphetamine and cocaine. However, in contrast to other amphetamine analogs, but similarly to other anorexiants such as benzphetamine (and its structural analog, BNMPA) and fenfluramine, 4-MAX has potent convulsant actions. Fenfluramine and p-chloroamphetamine produce a long-lasting depletion of 5-HT by inhibiting vesicle uptake and storage (Douglas, 1985). Bunker et al. (1990) noted that 4-MAX produced a substantial reduction in neostriatal TPH activity. They noted dramatic decreases in TPH activity in other brain regions (hippocampus and frontal cortex). They speculated that the early decline in 5-HT levels (30 minutes after 4-MAX administration) was due to release of the monamine and its subsequent metabolism while the decrease in 5-HT that occurred with 20 mg/kg at 3 hours after treatment reflected a decline in synthesis due to drug-induced TPH inhibition. They also noted that norepinephrine (NE) levels in the hippocampus were significantly lowered 3 hours after treatment with 4-MAX at 20 mg/kg. All animals in their 20 mg/kg group experienced convulsions within the first hour after treatment. The inhibitory role of NE in susceptibility to seizures, including electroconvulsions in mice, is well documented (Przegalinski, 1985).

1-[3-(Trifluoromethyl)phenyl] piperazine (TFMPP), a 5-HT agonist, has been shown to increase the convulsive threshold (be "anticonvulsant") in mice (Przegalinski, et al., 1994). They also demonstrated that the TFMPP-induced decrease in susceptibility to seizures is connected to stimulation of 5-HT_2 or of both 5-HT_{1C} and 5-HT_2 receptors. The anticonvulsant effect of TFMPP was

blocked by prazosin, an α_1 antagonist. It is not clear whether TFMPP can directly activate the noradrenergic system, however, electrical stimulation of the nucleus raphe dorsalis, which contains 5-HT cell bodies, has been shown to release 5-HT and enhance NE concentrations in the locus ceruleus (McRae-Degueurce, et al., 1985).

These models suggest some intriguing possibilities for the mechanism of BNMPA's convulsant action and some pathways for further investigation. BNMPA is structurally more similar to benzphetamine than either methamphetamine or amphetamine. Benzphetamine, like aminorex, 4-MAX and fenfluramine is a sympathomimetic amine that represented an attempt to produce a drug with anorectic properties while decreasing the central stimulant properties of this class of drug (Brooks, et al., 1982). In fact, among the major behavioral effects of stimulants such as amphetamine, the only one found to be mediated by a non-dopaminergic projection was anorexia (Robbins and Sahakian, 1983). When this structure-activity relationship is taken into consideration with the above described mechanisms of action of drugs in the stimulant class, we would predict that BNMPA would demonstrate more convulsant behavior than locomotor stimulation; in one reported case of benzphetamine poisoning (Brooks, et al., 1982), autopsy findings suggested a significant convulsant episode prior to death with the cause of death being circulatory collapse.

Even though BNMPA and its N-demethyl metabolite showed little affinity for the serotonin transporter, they, like their structurally analogous anorexiants, may inhibit TPH activity, cause direct cell death or be a 5-HT_{1C} or 5-HT_2 receptor antagonist. Since BNMPA also showed some affinity for the α_1-receptor, it may be acting as an antagonist at this site, thus contributing to its convulsant nature.

Additionally, it may be that genetic differences in receptor structure, function or density of stimulant binding sites in serotonergic, dopaminergic, muscarinic or sigma neuronal systems is what leads to variable individual sensitivity to seizures or death after stimulant administration.

As seems to be true with many scientific lines of inquiry, it is often the unexpected findings that may ultimately prove to be the most beneficial. Further investigation is warranted into the observation that BNMPA completely displaced CFT from the dopamine transporter and did not enhance dopamine release from striatal slices at concentrations that produced no alterations in locomotor activity ($\leq$ 100 μM) as well as its anticonvulsant potential via inhibition of NMDA-activated currents. BNMPA appears to have multiple sites of action in the CNS that could be important in modulating the behavioral effects of methamphetamine contaminated with this byproduct.

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List of References

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VITA

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MEMBERSHIP - SCIENTIFIC, HONORARY and PROFESSIONAL SOCIETIES

Phi Zeta (Veterinary Student Honor Society)
Phi Kappa Phi (Collegiate Scholastic Honor Society)
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AWARDS and HONORS

1975 - Tracy Bissette Memorial Scholarship for Academically Outstanding Women Veterinary Medical Students; Colorado State University

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PUBLICATIONS

1. Storz J, Moore KA, Spears P.: Use of Experimental Animals and "in vitro" Systems for the Detection and Identification of Chlamydial and Rickettsial Agents. International Symposium on Experimental Animals and "in vitro" Systems in Medical Microbiology; Munich, Federal Republic of Germany; September, 1976.

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PRESENTATIONS and PUBLISHED ABSTRACTS

1. Moore KA. Physical Characterization and GC/MS Analysis of α-Benzyl-N-methylphenethylamine (An Impurity of Illicit Methamphetamine Synthesis) in Urine. First Annual Pathology Student Research Seminar, Department of Pathology Grand Rounds, Medical College of Virginia, Richmond, Virginia, August 19, 1994.

2. Moore KA, Soine WH, Poklis A. Physical Characterization and GC/MS Analysis of α-Benzyl-N-methylphenethylamine (An Impurity of Illicit Methamphetamine Synthesis) in Urine. The International Association of Forensic Toxicologists (TIAFT) and Society of Forensic Toxicologists (SOFT) 1994 Joint Congress. Tampa, Florida, Oct. 31-Nov. 4, 1994.

3. Moore KA, Poklis A. Urinary Excretion of α-Benzyl-N-methylphenethylamine (An Impurity of Illicit Methamphetamine Synthesis) and Its Metabolites. American Academy of Forensic Sciences 47th Annual Meeting, Seattle, Washington, Feb. 13-19, 1995.

4. Moore KA, Soine WH, Borzelleca JF, Valentour JC, Saady JJ. The Analytical and Pharmacological Characterization of α-Benzyl-N-methylphenethylamine (BNMPA), An Impurity of Illicit Methamphetamine Synthesis. Society of Forensic Toxicologists Silver Anniversary Meeting, Baltimore, Maryland, October 9 - 14, 1995.